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(54) Title: TRANSFORMATION SYSTEM IN THE FIELD OF FILAMENTOUS FUNGAL HOSTS

(57) Abstract

A novel transformation system in the field of filamentous fungal hosts for expressing and secreting heterologous proteins or polypeptides is described. The invention also covers a process for producing large amounts of polypeptide or protein in an economical manner. The system comprises a transformed or transfected fungal strain of the genus *Chrysosporium*, more particularly of *Chrysosporium lucknowense* and mutants or derivatives thereof. It also covers transformants containing *Chrysosporium* coding sequences, as well as expression–regulating sequences of *Chrysosporium* genes.

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TRANSFORMATION SYSTEM IN THE FIELD OF FILAMENTOUS FUNGAL HOSTS

Summary of the invention

The subject invention pertains to a novel transformation system in the field of filamentous fungal hosts for expressing and secreting heterologous proteins or polypeptides. The invention also covers a process for producing large amounts of polypeptide in an economical manner. The system comprises a transformed or transfected fungal strain of the genus *Chrysosporium*, more particularly of *Chrysosporium lucknowense* and mutants or derivatives thereof. It also covers transformants containing *Chrysosporium* coding sequences. Novel mutant *Chrysosporium* strains are disclosed as are novel enzymes derived therefrom.

Background to the invention.

A number of hosts for gene expression and methods of transformation have been disclosed in the prior art. Bacteria are often mentioned e.g. *Escherichia coli*. *E. coli* is however a micro-organism incapable of secretion of a number of proteins or polypeptides and as such is undesirable as host cell for production of protein or polypeptide at the industrial level. An additional disadvantage for *E. coli*, which is valid also for bacteria in general, is that prokaryotes cannot provide additional modifications required for numerous eukaryotic proteins or polypeptides to be produced in an active form. Glycosylation of proteins and proper folding of proteins are examples of processing required to ensure an active protein or polypeptide is produced. To ensure such processing one can sometimes use mammalian cells; however, the disadvantage of such cells is that they are often difficult to maintain and require expensive media. Such transformation systems are therefore not practical for production of proteins or polypeptides at the industrial level. They may be cost efficient for highly priced pharmaceutical compounds requiring relatively low amounts, but certainly not for industrial enzymes.

A number of fungal expression systems have been developed e.g. *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus nidulans*, *Trichoderma reesei*. A number of others have been suggested but for various reasons have not found wide-spread acceptance or use. In general terms the ideal host must fulfil a large number of criteria:

- The ideal host must be readily fermented using inexpensive medium.
- The ideal host should use the medium efficiently.
- The ideal host must produce the polypeptide or protein in high yield, i.e. must exhibit high protein to biomass ratio.
- The ideal host should be capable of efficient secretion of the protein or polypeptide.
- The ideal host must enable ease of isolation and purification of the desired protein or polypeptide.
- The ideal host must process the desired protein or polypeptide such that it is produced in an active form not requiring additional activation or modification steps.

- The ideal host should be readily transformed.
- The ideal host should allow a wide range of expression regulatory elements to be used thus ensuring ease of application and versatility.
- The ideal host should allow use of easily selectable markers that are cheap to use.
- The ideal host should produce stable transformants.
- The ideal host should allow cultivation under conditions not detrimental to the expressed protein or polypeptide e.g. low viscosity, low shear.

Fungal systems that have not yet found widespread use are described e.g. in US Patent 5,578,463 by Berka et al suggesting *Neurospora, Podospora, Endothia, Mucor, Cochoibolus* and *Pyricularia* together with *Aspergillus* and *Trichoderma*. However only illustrations of transformation and expression are provided for *Aspergillus* and *Trichoderma* and no details are provided for any of the other suggested hosts.

WO 96/02563 and US patents 5,602,004, 5,604,129 and 5,695,985 to Novo Nordisk describe the drawbacks of *Aspergillus* and *Trichoderma* systems and suggests cultivation conditions for other fungi may be more suited to large scale protein production. The only examples provided for any transformed cultures are those of *Myceliophthora thermophila, Acremonium alabamense, Thielavia terrestris* and *Sporotrichum cellulophilum* strains. The *Sporotrichum* strain is reported to lyse and produce green pigment under fermentation conditions not leading to such results for the other strains. A non-sporulating mutant of *Thielavia terrestris* is described as being the organism of choice by virtue of its morphology. However it is also stated that the protoplasting efficiency of *Thielavia* and *Acremonium* (whereby the *Acremonium* strain used was the imperfect state of the *Thielavia* strain used) is low and that hygromycin was not useful as a selection marker. A large number of others are suggested as being potentially useful by virtue of their morphology but no transformation thereof is described. The suggested strains are *Corynascus, Thermoascus, Chaetomium, Ctenomyces, Scytalidium* and *Talaromyces*. The transformed hosts are mentioned as only producing low levels of the introduced *Humicola* xylanase with *Thielavia* producing the lowest amount; however, the information is ambiguous and could actually infer *Thielavia* was the best embodiment. The nomenclature of this reference is based on the ATCC names of Industrial Fungi of 1994. Thus it is apparent no high degree of heterologous expression was achieved and in fact no positive correlation could be derived between the postulated morphology and the degree of expression. If any correlation could be made, it was more likely to be negative. According to the 1996 ATCC fungal classification *Sporotrichum thermophilum* ATCC 20493 is a *Myceliophthora thermophila* strain. Currently the strain is still identified as *Myceliophthora thermophila*. The unpredicatability of the art is apparent from these recent disclosures.

Also Allison et al (*Curr. Genetics* 21:225-229, 1992) described transformation of *Humicola grisea* var. *thermoidea* using the lithium acetate method and a *Humicola* enzyme-encoding sequence,

but no report of expression of heterologous protein from such a strain has been provided.

In 1997 a patent issued to Hawaii Biotechnology Group for transformed *Neurospora* for expression of mammalian peptide such as chymosin. The transformation of auxotrophic *Neurospora crassa* occurred with spheroplasts. Endogenous transcriptional regulatory regions were introduced and cotransformation was carried out. Nothing is mentioned concerning other hosts and other transformation protocols. Nothing is apparent from the disclosure concerning the degree of expression. It is doubtful whether the degree of expression is high, as immunotechniques (which are useful for detecting small amounts of protein) are the only techniques used to illustrate the presence of the protein. No actual isolation of the protein is disclosed.

WO 97/26330 of Novo Nordisk suggests a method of obtaining mutants of filamentous fungal parent cells having an improved property for production of heterologous polypeptide. The method comprises first finding a specific altered morphology followed by assessing whether a transformant produces more heterologous polypeptide than the parent. The method is illustrated only for strains of *Fusarium* A3/5 and *Aspergillus oryzae*. The method is suggested to be applicable for *Aspergillus, Trichoderma, Thielavia, Fusarium, Neurospora, Acremonium, Tolyplocadium, Humicola, Scytalidium, Myceliophthora* or *Mucor*. As stated above the unpredictability in the art and also the unpredictability of the method of the cited application do not provide a generally applicable teaching with a reasonable expectation of success.

Detailed description of the invention.

We now describe an alternative fungal expression system with the simplicity of use of the above-mentioned *Aspergilli* and *Trichoderma* fulfilling the above requirements. The new system has not been taught or suggested in the prior art. The new system according to the invention provides the additional advantages that transformation rates are higher than those for the frequently used *Trichoderma reesei* system. In addition the culture conditions offer the additional bonus of being advantageous for the expressed polypeptide. Where reference is made in this specification and in the appending claims to "polypeptides" or "polypeptides of interest" as the products of the expression system of the invention, this term also comprise proteins, i.e polypeptides having a particular function and/or secondary and/or tertiary structure.

The pH of the culture medium can be neutral or alkaline thus no longer subjecting the produced protein or polypeptide to aggressive and potentially inactivating acid pH. It is also possible to culture at acid pH such as pH 4 for cases where the protein or polypeptide is better suited to an acidic environment. Suitably culture can occur at a pH between 4.0-10.0. A preference however exists for neutral to alkaline pH as the host strain exhibits better growth at such pH, e.g. between 6 and 9. Growth at alkaline temperature which can be from pH 8 up and can even be as high as 10 is also a good alternative for some cases. Also the cultivation temperature of such host strains is advantageous

to the stability of some types of produced polypeptide. The cultivation temperature is suitably at a temperature of 25-43°C. A temperature in the range from 40°C down to 23°C or 30°C is also advantageously applied. Clearly such conditions are of particular interest for production of mammalian polypeptides. The selected temperature will depend on cost effectiveness of the cultivation and sensitivity of the polypeptide or cultivation strain. The conditions will be determined by the skilled person without undue burden on a case-by-case basis, as is common in the art.

It has also been ascertained that the biomass to viscosity relation and the amount of protein produced is exceedingly favourable for the host according to the invention. Comparisons have been carried out with *Trichoderma longibrachiatum* (formerly also known as *Trichoderma reesei*) and with *Aspergillus niger*. *Trichoderma longibrachiatum* gave 2.5-5 g/l biomass, *Aspergillus niger* gave 5-10 g/l biomass and the host according to the invention gave 0.5-1 g/l biomass under their respective optimised conditions. This thus offers 5-10 fold improvement over the commercially used strains. These commercial strains are strains which themselves are considered in the art to be high producers of proteins and they are successfully used for commercial protein production. They have been cultured under their optimal conditions developed and run viably in large-scale commercial fermenters. The same strains were used to illustrate enormous improvement in viscosity values for cultures of the host according to the invention. At the end of the fermentation process *Trichoderma longibrachiatum* gave a value of 200-600 cP (Centipoise), *Aspergillus niger* gave a value of 1500-2000 cP and the host according to the invention gave a value below 10 cP. This thus provides at least 20-200 fold improvement for viscosity values over the commercially used strains. A quite surprising further aspect was that the protein levels determined for the host cells according to the invention were much higher than for the commercial *Aspergilli* and *Trichoderma reesei* strains, even with the above mentioned surprisingly low biomass and viscosity levels. In summary an easy to use versatile improved transformation system and expression system with improved culturing conditions has hereby been introduced. The strains according to the invention produce surprisingly higher protein levels under these improved conditions and in addition they do such in a shorter fermenter time.

The subject invention is directed at mutant Chrysosporium strains comprising a nucleic acid sequence encoding a heterologous protein or polypeptide, said nucleic acid sequence being operably linked to an expression regulating region and optionally a secretion signal encoding sequence and/or a carrier protein encoding sequence. Preferably a recombinant strain according to the invention will secrete the polypeptide of interest. This will avoid the necessity of disrupting the cell in order to isolate the polypeptide of interest and also minimise the risk of degradation of the expressed product by other components of the host cell.

Chrysosporium can be defined by morphology consistent with that disclosed in Barnett and Hunter 1972, Illustrated Genera of Imperfect Fungi, 3rd Edition of Burgess Publishing Company. Other sources providing details concerning classification of fungi of the genus *Chrysosporium* are

known e.g. Sutton Classification (Van Oorschot, C.A.N. (1980) "A revision of *Chrysosporium* and allied genera" in Studies in Mycology No. 20 of the CBS in Baarn The Netherlands p1-36). CBS is one of the depository institutes of the Budapest Treaty. According to these teachings the genus *Chrysosporium* falls within the family *Moniliaceae* which belongs to the order *Hyphomycetales*. The criteria that can be used are the following:

1. Signs of *Hyphomycetales* order:

Conidia are produced directly on mycelium, on separate sporogenous cells or on distinct conidiophores.

2. Signs of *Moniliaceae* family:

Both conidia and conidiophores (if present) are hyaline or brightly coloured; conidiophores are single or in loose clusters.

3. Signs of *Chrysosporium* Corda 1833 genus:

Colonies are usually spreading, white, sometimes cream-coloured, pale brown or yellow, felty and/or powdery. Hyphae are mostly hyaline and smooth-walled, with irregular, more or less orthotopic branching. Fertile hyphae exhibit little or no differentiation. Conidia are terminal and lateral, thallic, borne all over the hyphae, sessile or on short protrusions or side branches, subhyaline or pale yellow, thin- or thick-walled, subglobose, clavate, pyriform, orobovoid, 1-celled, rarely 2-celled, truncate. Intercalary conidia are sometimes present, are solitary, occasionally catenate, subhyaline or pale yellow, broader than the supporting hyphae, normally 1-celled, truncate at both ends. Chlamydospores are occasionally present.

Another source providing information on fungal nomenclature is ATCC (US). Their website is http://www.atcc.org. CBS also has a website (http//www.cbs.knaw.nl) providing relevant information. VKM in Moscow is also a reliable source of such information; the e-mail address for VKM is http://www.bdt.org.br.bdt.msdn.vkm/general. Another source is http//NT.ars-grin.gov/-fungaldatabases. All these institutions can provide teaching on the distinguishing characteristics of a *Chrysosporium*.

Strains defined as being of *Myceliophthora thermophila* are not considered to define *Chrysosporium* strains according to the definition of the invention. In the past there has been considerable confusion over the nomenclature of some *Myceliophthora* strains. Preferably the *Chrysosporium* according to the invention are those which are clearly distinguishable as such and cannot be confused with *Myceliophthora, Sporotrichum* or *Phanerochaete chrysosporium*.

The following strains are defined as *Chrysosporium* but the definition of *Chrysosporium* is not limited to these strains: *C. botryoides, C. carmichaelii, C. crassitunicatum, C. europae, C. evolceannui, C. farinicola, C. fastidium, C. filiforme, C. georgiae, C. globiferum, C. globiferum* var. *articulatum, C. globiferum* var. *niveum, C. hirundo, C. hispanicum, C. holmii, C. indicum, C. inops, C.*

keratinophilum, C. kreiselii, C. kuzurovianum, C. lignorum, C. lobatum, C. lucknowense, C. lucknowense Garg 27K, *C. medium, C. medium* var. *spissescens, C. mephiticum, C. merdarium, C. merdarium* var. *roseum, C. minor, C. pannicola, C. parvum, C. parvum* var. *crescens, C. pilosum, C. pseudomerdarium, C. pyriformis, C. queenslandicum, C. sigleri, C. sulfureum, C. synchronum, C. tropicum, C. undulatum, C. vallenarense, C. vespertilium, C. zonatum.*

C. lucknowense forms one of the species of *Chrysosporium* that have raised particular interest as it has provided a natural high producer of cellulase proteins (WO 98/15633 and related US 5,811,381). The characteristics of this *Chrysosporium lucknowense* are:

Colonies attain 55 mm diameter on Sabouraud glucose agar in 14 days, are cream-coloured, felty and fluffy; dense and 3-5 mm high; margins are defined, regular, and fimbriate; reverse pale yellow to cream-coloured. Hyphae are hyaline, smooth- and thin-walled, little branched. Aerial hyphae are mostly fertile and closely septate, about 1-3.5 mm wide. Submerged hyphae are infertile, about 1-4.5 mm wide, with the thinner hyphae often being contorted. Conidia are terminal and lateral, mostly sessile or on short, frequently conical protrusions or short side branches. Conidia are solitary but in close proximity to one another, 1-4 conidia developing on one hyphal cell, subhyaline, fairly thin- and smooth-walled, mostly subglobose, also clavate orobovoid, 1-celled, 2.5 x 11 x 1.5-6 mm, with broad basal scars (1-2 mm). Intercalary conidia are absent. Chlamydospores are absent. ATCC 44006, CBS 251.72, CBS 143.77 and CBS 272.77 are examples of *Chrysosporium lucknowense* strains and other examples are provided in WO 98/15633 and US 5,811,381.

A further strain was isolated from this species with an even higher production capacity for cellulases. This strain is called C1 by its internal notation and was deposited with the International Depository of the All Russian Collection of micro-organisms of the Russian Academy of Sciences Bakrushina Street 8, Moscow, Russia 113184 on August 29, 1996, as a deposit according to the Budapest Treaty and was assigned Accession Number VKM F-3500D. It is called *Chrysosporium lucknowense* Garg 27K. The characteristics of the C1 strain are as follows:

Colonies grow to about 55-66 mm diameter in size on potato-dextrose agar in about 7 days; are white-cream-coloured, felty, 2-3 mm high at the center; margins are defined, regular, fimbriate; reverse pale, cream-coloured. Hyphae are hyaline, smooth- and thin-walled, little branched. Aerial hyphae are fertile, septate, 2-3 mm wide. Submerged hyphae are infertile. Conidia are terminal and lateral; sessile or on short side branches; absent; solitary, but in close proximity to one another, hyaline, thin- and smooth-walled, subglobose, clavate or obovoid, 1-celled, 4-10 mm. Chlamydospores are absent. Intercalary conidia are absent.

The method of isolation of the C1 strain is described in WO 98/15633 and US 5,811,381. Strains exhibiting such morphology are included within the definition of *Chrysosporium* according to the invention. Also included within the definition of *Chrysosporium* are strains derived from *Chrysosporium* predecessors including those that have mutated somewhat either naturally or by induced

mutagenesis. In particular the invention covers mutants of *Chrysosporium* obtained by induced mutagenis, especially by a combination of irradiation and chemical mutagenesis.

For example strain C1 was mutagenised by subjecting it to ultraviolet light to generate strain UV13-6. This strain was subsequently further mutated with N-methyl-N'-nitro-N-nitrosoguanidine to generate strain NG7C-19. The latter strain in turn was subjected to mutation by ultraviolet light resulting in strain UV18-25. During this mutation process the morphological characteristics have varied somewhat in culture in liquid or on plates as well as under the microscope. With each successive mutagenesis the cultures showed less of the fluffy and felty appearance on plates that are described as being characteristic of *Chrysosporium*, until the colonies attained a flat and matted appearance. A brown pigment observed with the wild type strain in some media was also less prevalent in mutant strains. In liquid culture the mutant UV18-25 was noticeably less viscous than the wild type strain C1 and the mutants UV13-6 and NG7C-19. While all strains maintained the gross microscopic characteristics of *Chrysosporium*, the mycelia became narrower with each successive mutation and with UV18-25 distinct fragmentation of the mycelia could be observed. This mycelial fragmentation is likely to be the cause of the lower viscosity associated with cultures of UV18-25. The ability of the strains to sporulate decreased with each mutagenic step. The above illustrates that for a strain to belong to the genus *Chrysosporium* there is some leeway from the above morphological definition. At each mutation step production of cellulase and extracellular proteins has in addition also increased, while several mutations resulted in decrease of protease expression. Criteria with which fungal taxonomy can be determined are available from CBS, VKMF and ATCC for example.

In particular the anamorph form of *Chrysosporium* has been found to be suited for the production application according to the invention. The metabolism of the anamorph renders it extremely suitable for a high degree of expression. A teleomorph should also be suitably as the genetic make-up of the anamorphs and teleomorphs is identical. The difference between anamorph and teleomorph is that one is the asexual state and the other is the sexual state. The two states exhibit different morphology under certain conditions.

It is preferable to use non-toxic *Chrysosporium* strains of which a number are known in the art as this will reduce risks to the environment upon large scale production and simplify production procedures with the concomitant reduction in costs.

An expression-regulating region is a DNA sequence recognised by the host *Chrysosporium* strain for expression. It comprises a promoter sequence operably linked to a nucleic acid sequence encoding the polypeptide to be expressed. The promoter is linked such that the positioning vis-à-vis the initiation codon of the sequence to be expressed allows expression. The promoter sequence can be constitutive or inducible. Any expression regulating sequence or combination thereof capable of permitting expression of a polypeptide from a *Chrysosporium* strain is envisaged. The expression regulating sequence is suitably a fungal expression-regulating region e.g. an ascomycete regulating

region. Suitably the fungal expression regulating region is a regulating region from any of the following genera of fungi: *Aspergillus, Trichoderma, Chrysosporium, Hansenula, Mucor, Pichia, Neurospora, Tolypocladium, Rhizomucor, Fusarium, Penicillium, Saccharomyces, Talaromyces* or alternative sexual forms thereof like *Emericella, Hypocrea* e.g. the cellobiohydrolase promoter from *Trichoderma*, glucoamylase promoter from *Aspergillus*, glyceraldehyde phosphate dehydrogenase promoter from *Aspergillus*, alcohol dehydrogenase A and alcohol dehydrogenase R promoter of *Aspergillus*, TAKA amylase promoter from *Aspergillus*, phosphoglycerate and cross-pathway control promoters of *Neurospora*, aspartic proteinase promoter of *Rhizomucor miehei*, lipase promoter of *Rhizomucor miehei* and beta-galactosidase promoter of *Penicillium canescens*. An expression regulating sequence from the same genus as the host strain is extremely suitable, as it is most likely to be specifically adapted to the specific host. Thus preferably the expression regulating sequence is one from a *Chrysosporium* strain.

We have found particular strains of *Chrysosporium* to express proteins in extremely large amounts and natural expression regulating sequences from these strains are of particular interest. These strains are internally designated as *Chrysosporium* strain C1, strain UV13-6, strain NG7C-19 and strain UV18-25. They have been deposited in accordance with the Budapest Treaty with the All Russian Collection (VKM) depository institute in Moscow. Wild type C1 strain was deposited in accordance with the Budapest Treaty with the number VKM F-3500 D, deposit date 29-08-1996, C1 UV13-6 mutant was deposited with number VKM F-3632 D, and deposit date 02-09-1998, C1 NG7c-19 mutant was deposited with number VKM F-3633 D and deposit date 02-09-1998 and C1 UV18-25 mutant was deposited with number VKM F-3631 D and deposit date 02-09-1998.

Preferably an expression-regulating region enabling high expression in the selected host is applied. This can also be a high expression-regulating region derived from a heterologous host, such as are well known in the art. Specific examples of proteins known to be expressed in large quantities and thus providing suitable expression regulating sequences for the invention are without being limited thereto hydrophobin, protease, amylase, xylanase, pectinase, esterase, beta-galactosidase, cellulase (e.g. endo-glucanase, cellobiohydrolase) and polygalacturonase. The high production has been ascertained in both solid state and submerged fermentation conditions. Assays for assessing the presence or production of such proteins are well known in the art. The catalogues of Sigma and Megazyme for example provide numerous examples. Megazyme is located at Bray Business Park, Bray, County Wicklow in Ireland. Sigma Aldrich has many affiliates world wide e.g. USA P.O. Box 14508 St. Louis Missouri. For cellulase we refer to commercially available assays such as CMCase assays, endoviscometric assays, Avicelase assays, beta-glucanase assays, RBBCMCase assays, Cellazyme C assays. Alternatives are well known to a person skilled in the art and can be found from general literature concerning the subject and such information is considered incorporated herein by reference. By way of example we refer to "Methods in Enzymology Volume 1, 1955 right through to

volumes 297-299 of 1998. Suitably a *Chrysosporium* promoter sequence is applied to ensure good recognition thereof by the host.

We have found that heterologous expression-regulating sequences work as efficiently in *Chrysosporium* as native *Chrysosporium* sequences. This allows well known constructs and vectors to be used in transformation of *Chrysosporium* as well as offering numerous other possibilities for constructing vectors enabling good rates of expression in this novel expression and secretion host. For example standard *Aspergillus* transformation techniques can be used as described for example by Christiansen et al in Bio/Technol. 6:1419-1422 (1988). Other documents providing details of *Aspergillus* transformation vectors, e.g. US patents 4,816,405, 5,198,345, 5,503,991, 5,364,770 and 5,578,463, EP-B-215.594 (also for *Trichoderma*) and their contents are incorporated by reference. As extremely high expression rates for cellulase have been ascertained for *Chrysosporium* strains, the expression regulating regions of such proteins are particularly preferred. We refer for specific examples to the previously mentioned deposited *Chrysosporium* strains.

A nucleic acid construct comprising a nucleic acid expression regulatory region from *Chrysosporium*, preferably from *Chrysosporium lucknowense* or a derivative thereof forms a separate embodiment of the invention as does the mutant *Chrysosporium* strain comprising such operably linked to a polypeptide to be expressed. Suitably such a nucleic acid construct will be an expression regulatory region from *Chrysosporium* associated with cellulase or xylanase expression, preferably cellobiohydrolase expression, more specifically expression of a 55 kDa cellobiohydrolase. The *Chrysosporium* promoter sequences of an endoglucanase of 25 kDa (C1-EG5) and of an endoglucanase of 43 kDa (C1-EG6), wherein the molecular weights are determined according to SDS PAGE (with the molecular weights according to amino acid sequence data being 21.9 kDa and 39.5 kDa), are provided by way of example. Thus, the *Chrysosporium* promoter sequences of hydrophobin, protease, amylase, xylanase, esterase, pectinase, beta-galactosidase, cellulase (e.g. endoglucanase, cellobiohydrolase) and polygalacturonase are considered to also fall within the scope of the invention. Any of the promoters or regulatory regions of expression of enzymes disclosed in Table A or B can be suitably employed. The nucleic acid sequence according to the invention can suitably be obtained from a *Chrysosporium* strain according to the invention, such strain being defined elsewhere in the description. The manner in which promoter sequences can be determined are numerous and well known in the art. Nuclease deletion experiments of the region upstream of the ATG codon at the beginning of the relevant gene will provide such sequence. Also for example analysis of consensus sequences can lead to finding a gene of interest. Using hybridisation and amplification techniques one skilled in the art can readily arrive at the corresponding promoter sequences.

The promoter sequences of C1 endoglucanases were identified in this manner, by cloning the corresponding genes, and are given in SEQ ID No.'s 2 (EG5) and 1 (EG6), respectively. Other preferred promoters according to the invention are the 55 kDa cellobiohydrolase (CBH1) promoter

and the 30 kDa xylanase (XylF) promoters, as the enzymes are expressed at high level by their own promoters. The corresponding promoter sequences can be identified in a straightforward manner by cloning as described below for the endoglucanase promoters, using the partial sequence information given in SEQ ID No. 4 (for CBH1) and SEQ ID No. 5 (for XylF), respectively. The promoters of the carbohydrate-degrading enzymes of *Chrysosporium*, especially C1 promoters, can advantageously be used for expressing desired polypeptides in a host organism, especially a fungal or other microbial host organism. Promoter sequences having at least 60%, preferably at least 70%, most preferably at least 80% nucleotide sequence identity with the sequence given in SEQ ID No's 1 and 2, or with the sequences found for other *Chrysosporium* genes, are part of the present invention.

For particular embodiments of the recombinant strain and the nucleic acid sequence according to the invention we also refer to the examples. We also refer for the recombinant strains to prior art describing high expression promoter sequences in particular those providing high expression in fungi e.g. such as are disclosed for *Aspergillus* and *Trichoderma*. The prior art provides a number of expression regulating regions for use in *Aspergillus* e.g. US 5,252,726 of Novo and US 5,705,358 of Unilever. The contents of such prior art are hereby incorporated by reference.

The hydrophobin gene is a fungal gene that is highly expressed. It is thus suggested that the promoter sequence of a hydrophobin gene, preferably from *Chrysosporium,* may be suitably applied as expression regulating sequence in a suitable embodiment of the invention. *Trichoderma reesei* and *Trichoderma harzianum* gene sequences for hydrophobin have been disclosed for example in the prior art as well as a gene sequence for *Aspergillus fumigatus* and *Aspergillus nidulans* and the relevant sequence information is hereby incorporated by reference (Munoz et al, *Curr. Genet.* 1997, 32(3):225-230; Nakari-Setala T. et al, *Eur. J. Biochem.* 1996 15:235 (1-2):248-255, M. Parta et al, *Infect. Immun.* 1994 62 (10): 4389-4395 and Stringer M.A. et al. *Mol. Microbiol.* 1995 16(1):33-44). Using this sequence information a person skilled in the art can obtain the expression regulating sequences of *Chrysosporium* hydrophobin genes without undue experimentation following standard techniques as suggested already above. A recombinant *Chrysosporium* strain according to the invention can comprise a hydrophobin-regulating region operably linked to the sequence encoding the polypeptide of interest.

An expression regulating sequence can also additionally comprise an enhancer or silencer. These are also well known in the prior art and are usually located some distance away from the promoter. The expression regulating sequences can also comprise promoters with activator binding sites and repressor binding sites. In some cases such sites may also be modified to eliminate this type of regulation. Filamentous fungal promoters in which creA sites are present have been described. Such creA sites can be mutated to ensure the glucose repression normally resulting from the presence of the non-mutated sites is eliminated. Gist-Brocades' WO 94/13820 illustrates this principle. Use of such a promoter enables production of the polypeptide encoded by the nucleic acid sequence regulated

by the promoter in the presence of glucose. The same principle is also apparent from WO 97/09438. These promoters can be used either with or without their creA sites. Mutants in which the creA sites have been mutated can be used as expression regulating sequences in a recombinant strain according to the invention and the nucleic acid sequence it regulates can then be expressed in the presence of glucose. Such *Chrysosporium* promoters ensure derepression in an analogous manner to that illustrated in WO 97/09438. The identity of creA sites is known from the prior art. Alternatively, it is possible to apply a promoter with CreA binding sites that have not been mutated in a host strain with a mutation elsewhere in the repression system e.g. in the creA gene itself, so that the strain can, notwithstanding the presence of creA binding sites, produce the protein or polypeptide in the presence of glucose.

Terminator sequences are also expression-regulating sequences and these are operably linked to the 3' terminus of the sequence to be expressed. Any fungal terminator is likely to be functional in the host *Chrysosporium* strain according to the invention. Examples are *A. nidulans* trpC terminator (1), *A. niger* alpha-glucosidase terminator (2), *A. niger* glucoamylase terminator (3), *Mucor miehei* carboxyl protease terminator (US 5,578,463) and the *Trichoderma reesei* cellobiohydrolase terminator. Naturally *Chrysosporium* terminator sequences will function in *Chrysosporium* and are suitable e.g. EG6 terminator.

A suitable recombinant *Chrysosporium* strain according to the invention has the nucleic acid sequence to be expressed operably linked to a sequence encoding the amino acid sequence defined as signal sequence. A signal sequence is an amino acid sequence which when operably linked to the amino acid sequence of the expressed polypeptide allows secretion thereof from the host fungus. Such a signal sequence may be one normally associated with the heterologous polypeptide or may be one native to the host. It can also be foreign to both host and the polypeptide. The nucleic acid sequence encoding the signal sequence must be positioned in frame to permit translation of the signal sequence and the heterologous polypeptide. Any signal sequence capable of permitting secretion of a polypeptide from a *Chrysosporium* strain is envisaged. Such a signal sequence is suitably a fungal signal sequence, preferably an ascomycete signal sequence.

Suitable examples of signal sequences can be derived from yeasts in general or any of the following specific genera of fungi: *Aspergillus, Trichoderma, Chrysosporium, Pichia, Neurospora, Rhizomucor, Hansenula, Humicola, Mucor, Tolypocladium, Fusarium, Penicillium, Saccharomyces, Talaromyces* or alternative sexual forms thereof like *Emericella, Hypocrea*. Signal sequences that are particularly useful are often natively associated with the following proteins a cellobiohydrolase, an endoglucanase, a beta-galactosidase, a xylanase, a pectinase, an esterase, a hydrophobin, a protease or an amylase. Examples include amylase or glucoamylase of *Aspergillus* or *Humicola* (4), TAKA amylase of *Aspergillus oryzae*, alpha-amylase of *Aspergillus niger*, carboxyl peptidase of *Mucor* (US 5,578,463), a lipase or proteinase from *Rhizomucor miehei*, cellobiohydrolase of *Trichoderma* (5),

beta-galactosidase of *Penicillium canescens* and alpha mating factor of *Saccharomyces*.

Alternatively the signal sequence can be from an amylase or subtilisin gene of a strain of *Bacillus*. A signal sequence from the same genus as the host strain is extremely suitable as it is most likely to be specifically adapted to the specific host thus preferably the signal sequence is a signal sequence of *Chrysosporium*. We have found particular strains of *Chrysosporium* to excrete proteins in extremely large amounts and naturally signal sequences from these strains are of particular interest. These strains are internally designated as *Chrysosporium* strain C1, strain UV13-6, strain NG7C-19 and strain UV18-25. They have been deposited in accordance with the Budapest Treaty as described elsewhere in this description. Signal sequences from filamentous fungi, yeast and bacteria are useful. Signal sequences of non-fungal origin are also considered useful, particularly bacterial, plant and mammalian.

A recombinant *Chrysosporium* strain according to any of the embodiments of the invention can further comprise a selectable marker. Such a selectable marker will permit easy selection of transformed or transfected cells. A selectable marker often encodes a gene product providing a specific type of resistance foreign to the non-transformed strain. This can be resistance to heavy metals, antibiotics and biocides in general. Prototrophy is also a useful selectable marker of the non-antibiotic variety. Non-antibiotic selectable markers can be preferred where the protein or polypeptide of interest is to be used in food or pharmaceuticals with a view to speedier or less complicated regulatory approval of such a product. Very often the GRAS indication is used for such markers. A number of such markers are available to the person skilled in the art. The FDA e.g. provides a list of such. Most commonly used are selectable markers selected from the group conferring resistance to a drug or relieving a nutritional defect e.g the group comprising amdS (acetamidase), hph (hygromycin phosphotransferase), pyrG (orotidine-5'-phosphate decarboxylase), trpC (anthranilate synthase), argB (ornithine carbamoyltransferase), sC (sulphate adenyltransferase), bar (phosphinothricin acetyl-transferase), glufosinate resistance, niaD (nitrate reductase), a bleomycin resistance gene, more specifically Sh ble, sulfonylurea resistance e.g. acetolactate synthase mutation ilv1. Selection can also be carried out by virtue of cotransformation where the selection marker is on a separate vector or where the selection marker is on the same nucleic acid fragment as the polypeptide-encoding sequence for the polypeptide of interest.

As used herein the term heterologous polypeptide is a protein or polypeptide not normally expressed and secreted by the *Chrysosporium* host strain used for expression according to the invention. The polypeptide can be of plant or animal (vertebrate or invertebrate) origin e.g. mammalian, fish, insect, or micro-organism origin, with the proviso it does not occur in the host strain. A mammal can include a human. A micro-organism comprises viruses, bacteria, archaebacteria and fungi i.e. filamentous fungi and yeasts. Bergey's Manual for Bacterial Determinology provides adequate lists of bacteria and archaebacteria. For pharmaceutical purposes quite often a preference

will exist for human proteins thus a recombinant host according to the invention forming a preferred embodiment will be a host wherein the polypeptide is of human origin. For purposes such as food production suitably the heterologous polypeptide will be of animal, plant or algal origin. Such embodiments are therefore also considered suitable examples of the invention. Alternative embodiments that are useful also include a heterologous polypeptide of any of bacterial, yeast, viral, archaebacterial and fungal origin. Fungal origin is most preferred.

A suitable embodiment of the invention will comprise a heterologous nucleic acid sequence with adapted codon usage. Such a sequence encodes the native amino acid sequence of the host from which it is derived, but has a different nucleic acid sequence, i.e. a nucleic acid sequence in which certain codons have been replaced by other codons encoding the same amino acid but which are more readily used by the host strain being used for expression. This can lead to better expression of the heterologous nucleic acid sequence. This is common practice to a person skilled in the art. This adapted codon usage can be carried out on the basis of known codon usage of fungal vis-à-vis non-fungal codon usage. It can also be even more specifically adapted to codon usage of *Chrysosporium* itself. The similarities are such that codon usage as observed in *Trichoderma, Humicola* and *Aspergillus* should enable exchange of sequences of such organisms without adaptation of codon usage. Details are available to the skilled person concerning the codon usage of these fungi and are incorporated herein by reference.

The invention is not restricted to the above mentioned recombinant *Chrysosporium* strains, but also covers a recombinant *Chrysosporium* strain comprising a nucleic acid sequence encoding a homologous protein for a *Chrysosporium* strain, said nucleic acid sequence being operably linked to an expression-regulating region and said recombinant strain expressing more of said protein than the corresponding non-recombinant strain under the same conditions. In the case of homologous polypeptide of interest such is preferably a neutral or alkaline enzyme like a hydrolase, a protease or a carbohydrate degrading enzyme as already described elsewhere. The polypeptide may also be acidic. Preferably the recombinant strain will express the polypeptide in greater amounts than the non-recombinant strain. All comments mentioned vis-à-vis the heterologous polypeptide are also valid (mutatis mutandis) for the homologous polypeptide cellulase.

Thus the invention also covers genetically engineered *Chrysosporium* strains wherein the sequence that is introduced can be of *Chrysosporium* origin. Such a strain can, however, be distinguished from natively occurring strains by virtue of for example heterologous sequences being present in the nucleic acid sequence used to transform or transfect the *Chrysosporium*, by virtue of the fact that multiple copies of the sequence encoding the polypeptide of interest are present or by virtue of the fact that these are expressed in an amount exceeding that of the non-engineered strain under identical conditions or by virtue of the fact that expression occurs under normally non-expressing conditions. The latter can be the case if an inducible promoter regulates the sequence of interest

contrary to the non-recombinant situation or if another factor induces the expression than is the case in the non-engineered strain. The invention as defined in the preceding embodiments is not intended to cover naturally occurring *Chrysosporium* strains. The invention is directed at strains derived through engineering either using classical genetic technologies or genetic engineering methodologies.

All the recombinant strains of the invention can comprise a nucleic acid sequence encoding a heterologous protein selected from carbohydrate-degrading enzymes (cellulases, xylanases, mannanases, mannosidases, pectinases, amylases, e.g. glucoamylases, α-amylases, alpha- and beta-galactosidases, α- and β-glucosidases, β-glucanases, chitinases, chitanases), proteases (endoproteases, amino-proteases, amino-and carboxy-peptidases), other hydrolases (lipases, esterases, phytases), oxidoreductases (catalases, glucose-oxidases) and transferases (transglycosylases, transglutaminases, isomerases and invertases).

Table A: pH range where enzymes retain activity and/or stability

Sample	pH range retaining > 50% enzymatic activity			pH range retaining > 70% enzymatic activity			Stability (20h,50°C) % from max
	CMC ase	RBB-CMC-ase	Other sub-strates	CMC-ase	RBB-CMC ase	Other sub-strates	pH 7.5/8
30 Kd protease (alkaline) 30 kD	-	-	12.5	-	-	12.0	-
Xyl (alkaline)	-	-	10.0	-	-	8.5	80
51 kD Xyl	-	-	8.0	-	-	7.5	-
60 kD Xyl	-	-	9.5	-	-	9.0	85
45 kD endo	7.0	8.0	-	6.5	7.0	-	75
55 kD endo	8.0	8.0	-	7.0	7.0	-	55
25 kD(21.8 kD*)endo	7,5	10.0	-	6.5	9.0	-	80
43 kD(39.6 kD*)endo	8.0	8.0	-	7.2	7.2	-	-
45 kD α,ß-Gal/ß-Gluc	-	-	6.8	-	-	5.7	-
48 kD CBH with ß-Gluc traces	5.2	7.5	8.0	5.0	6.8	-	-
55 kD CBH	8.0	9.0	-	7.4	8.5	-	70
65 kD PGU	-	-	8.0	-	-	7.3	-
90 kD protease	-	-	9.0	-	-	9.0	-
100 kD esterase	-	-	9.0	-	-	9.0	-

* molecular weights (by MALDI)

Note:
* all other molecular weights by SDS PAGE
* enzymes were taken in equal protein contents
* xyl = xylanase
* endo = endoglucanase
* gal = galactosidase
* gluc = glucosidase
* CBN = cellbiohydrolase
* PGU = polygalacturonase

Table B. Activities of enzymes isolated from ultrafiltrate from 18-25 strain toward different substrates (pH 5), units/mg protein

Sample	pI	CMC	RBB-CMC	CMC-41	FP	CMC (visc)	b-Glu-can	pNP-a-G	pNP-b-G	Cello-biose	Avicel	MUF-cello-biosid e	MUF-lac-toside	MUF-xylo side	Lactos e	Xylan	Polyg a-lactur onic acid	MUF-gluco-side	Galact o-manna n	pNP-a-galact oside	pNP-b-galact oside	Dyed casein **	pNP buryra te
		50oC	40oC	40oC	50oC	40oC	50oC	40oC	40oC	40oC	40oC	40oC	40oC	40oC	40oC	50oC	50oC	40oC	50oC	40oC	40oC	50oC	60oC
30 kD protease	8.9	0	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4	0
30 kD Xyl	9.1	0.1	2	0.1	0.16	0.1	0	-	0	-	0	0	0	0	-	25	0	0	0	0	-	0	0
51 kD Xyl	8.7	0.1	4.2	-	0.19	-	0	-	0	-	0	0	0	0	-	19	0	0	0	0	-	0	0
60 kD Xyl	4.7	0	-	-	0	-	0	-	0	-	0	0.14	0.02	0.04	-	16.3	0	0	0	0	0	0	0
45 kD endo	6	51	86	7.6	0.2	47	36	-	0	-	0.5	0	0	0	-	1	-	0	1.8	0	-	0	0
55 kD endo	4.9	47	94	7.7	0.3	39	25	-	0	-	0.5	0	0	0	-	0	-	0	0.4	0	-	0	0
25 kD (21.8 kD*) endo	4.1	19	15	3.9	0.3	11	3.8	-	0	0	0.05	0	0	-	0	0.03	0	-	0	0	0	0	0
43 kD (39.6 kD*) endo	4.2	0.43	0.2	0.1	0	0.2	0.2	-	0	0	0	0	0	-	0	0	0	-.	0	0	0	0	0
45 kD a,b-Gal/b-Gluc	4.2	0	0	0	0	0.01	0.01	0	0.4	0.06	0	0	0	-	0.01	0	0.1	0.1	0.2	0.2	0.3	0	1.7
~~40 kD CBH with b-Gluc~~ traces+ glucono-d-lactone	4.4	0.67	1.3	1.2	0.4	0.8	0.77	0	1.7	0.08	0	0.2	0.36	-	0	0	0.1	0.4	0	0	0	0	2.3
									0				0.36										
55 kD CBH with b-Gluc traces + glucono-d-lactone	4.4	0.7	0.16	0.27	0.4	0.1	0.1	-	0.05	0.08	0.46	0.2	0.7	-	0	0.1	0	-	0	0	0	0	0
									0			0.14	0.6										
65 kD PGU	4.4	0	0	0	0	0	0	-	0	0	0	0	0	-	0	0	1	-	0	0	0	0	0
90 kD protease	4.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-
100 kD esterase	4.5	0	0	0	0	0	0	-	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0.8

*molecular weights (by MALDI)
** activity toward dyed casein was expressed in arbitrary units/mg

The most interesting products to be produced accoprding to invention are cellulases, xylanases, pectinases, lipases and proteases, wherein cellulases and xylanases cleave beta-1,4-bonds, and cellulases comprise endoglucanases, cellobiohydrolases and beta-glucosidases. These proteins are extremely useful in various industrial processes known in the art. Specifically for cellulases we refer e.g. to WO 98/15633 describing cellobiohydrolases and endoglucanases of use. The contents of said application are hereby incorporated by reference. We also refer to Tables A and B providing further details of interesting *Chrysosporium* proteins.

It was found according to the invention, that *Chrysosporium* mutants can be made that have reduced expression of protease, thus making them even more suitable for the production of proteinaceous products, especially if the proteinaceous product is sensitive to protease activity. Thus the invention also involves a mutant *Chrysosporium* strain which produces less protease than non-mutant *Chrysosporium* strain, for example less than *C. lucknowense* strain C1 (VKM F-3500 D). In particular the protease acitivity of such strains is less than half the amount, more in particular less than 30% of the amount produced by C1 strain. The decreased protease activity can be measured by known methods, such as by measuring the halo formed op skim milk plates or BSA degradation.

An embodiment of the invention that is of particular interest is a recombinant *Chrysosporium* according to the invention wherein the nucleic acid sequence encoding the polypeptide of interest encodes a polypeptide that is inactivated or unstable at acid pH i.e. pH below 6, even below pH 5,5, more suitably even below pH 5 and even as low as or lower than pH 4. This is a particularly interesting embodiment, as the generally disclosed fungal expression systems are not cultured under conditions that are neutral to alkaline, but are cultured at acidic pH. Thus the system according to the invention provides a safe fungal expression system for proteins or polypeptides that are susceptible to being inactivated or are unstable at acid pH.

Quite specifically a recombinant strain as defined in any of the embodiments according to the invention, wherein the nucleic acid sequence encoding the polypeptide of interest encodes a protein or polypeptide exhibiting optimal activity and/or stability at a pH above 5, preferably at neutral or alkaline pH (i.e. above 7) and/or at a pH higher than 6, is considered a preferred embodiment of the invention. More than 50%, more than 70% and even more than 90% of optimal activities at such pH values are anticipated as being particularly useful embodiments. A polypeptide expressed under the cultivation conditions does not necessarily have to be active at the cultivation conditions, in fact it can be advantageous for it to be cultured under conditions under which it is inactive as its active form could be detrimental to the host. This is the case for proteases for example. What is however required is for the protein or polypeptide to be stable under the cultivation conditions. The stability can be thermal stability. It can also be stability against specific compositions or chemicals, such as are present for example in compositions or processes of production or

application of the polypeptide or protein of interest. LAS in detergent compositions comprising cellulases or lipases, etc. is an example of a chemical often detrimental to proteins. The time periods of use in applications can vary from short to long exposure so stability can be over a varying length of time varying per application. The skilled person will be able to ascertain the correct conditions on a case by case basis. One can use a number of commercially available assays to determine the optimal activities of the various enzymatic products. The catalogues of Sigma and Megazyme for example show such. Specific examples of tests are mentioned elsewhere in the description. The manufacturers provide guidance on the application.

We have surprisingly found that a *Chrysosporium* strain that can be suitably used to transform or transfect with the sequence of interest to be expressed is a strain exhibiting relatively low biomass. We have found that *Chrysosporium* strains having a biomass two to five times lower than that of *Trichoderma reesei* when cultured to a viscosity of 200-600 cP at the end of fermentation and exhibiting a biomass of 10 to 20 times lower than that of *Aspergillus niger* when cultured to a viscosity of 1500-2000 cP under corresponding conditions, i.e. their respective optimal cultivation conditions can provide a high level of expression. This level of expression far exceeds that of the two commercial reference strains at a much lower biomass and at much lower viscosity. This means that the yield of expression of such *Chrysosporium* strains will be appreciably higher than from *Aspergillus niger* and *Trichoderma reesei*. Such a transformed or transfected *Chrysosporium* strain forms a suitable embodiment of the invention.

We find a biomass of 0,5-1,0 g/l for *Chrysosporium* strain C1(18-25) as opposed to 2,5-5,0 g/l for *Trichoderma reesei* and 5-10 g/l of *Aspergillus niger* under the above described conditions. In the Examples we provide details of this process.

In a suitable embodiment a recombinant *Chrysosporium* strain according to the invention produces protein or polypeptide in at least the amount equivalent to the production in moles per liter of cellulase by the strain UV13-6 or C-19, and most preferably at least equivalent to or higher than that of the strain UV18-25 under the corresponding or identical conditions, i.e. their respective optimal cultivation conditions.

Unexpectedly we have also found that expression and secretion rates are exceedingly high when using a *Chrysosporium* strain exhibiting the mycelial morphology of strain UV18-25 i.e. fragmented short mycelia. Thus a recombinant strain according to the invention will preferably exhibit such morphology. The invention however also covers non-recombinant strains or otherwise engineered strains of *Chrysosporium* exhibiting this novel and inventive characteristic. Also covered by the invention is a recombinant *Chrysosporium* strain in any of the embodiments described according to the invention further exhibiting reduced sporulation in comparison to C1, preferably below that of strain UV13-6, preferably below that of NG7C-19, preferably below that of UV18-25 under equivalent fermenter conditions. Also covered by the invention is a recombinant *Chrysosporium*

strain in any of the embodiments described according to the invention further exhibiting at least the amount of protein production ratio to biomass in comparison to C1, preferably in comparison to that of any of strains UV13-6, NG7C-19 and UV18-25 under equivalent fermenter conditions. The invention however also covers non-recombinant strains or otherwise engineered strains of *Chrysosporium* exhibiting this novel and inventive characteristic as such or in combination with any of the other embodiments.

Another attractive embodiment of the invention also covers a recombinant *Chrysosporium* strain exhibiting a viscosity below that of strain NG7C-19, preferably below that of UV18-25 under corresponding or identical fermenter conditions. The invention however also covers non-recombinant strains or otherwise engineered strains of *Chrysosporium* exhibiting this novel and inventive characteristic as such or in combination with any of the other embodiments. We have determined that the viscosity of a culture of UV18-25 is below 10 cP opposed to that of *Trichoderma reesei* being of the order 200-600 cP, with that of *Aspergillus niger* being of the order 1500-2000 cP under their respective optimal culture conditions at the end of fermentation. The process used for such determination is provided in the examples.

Viscosity can be assessed in many cases by visual monitoring. The fluidity of the substance can vary to such a large extent that it can be nearly solid, sauce like or liquid. Viscosity can also readily be ascertained by Brookfield rotational viscometry, use of kinematic viscosity tubes, falling ball viscometer or cup type viscometer. The yields from such a low viscosity culture are higher than from the commercial known higher viscosity cultures per time unit and per cell.

The processing of such low viscosity cultures according to the invention is advantageous in particular when the cultures are scaled up. The subject *Chrysosporium* strains with the low viscosity perform very well in cultures as large as up to 150,000 liter cultures. Thus any culture size up to 150,000 litres provides a useful embodiment of the invention. Any other conventional size of fermentation should be carried out well with the strains according to the invention. The reasoning behind this is that problems can arise in large scale production with the formation of aggregates that have mycelia that are too dense and/or are unevenly distributed. The media as a result cannot be effectively utilised during the culture thus leading to an inefficient production process in particular in large scale fermentations i.e. over 150,000 liters. Aeration and mixing become problematic leading to oxygen and nutrient starvation and thus reduced concentration of productive biomass and reduced yield of polypeptide during the culture and/or can result in longer fermentation times. In addition high viscosity and high shear are not desirable in commercial fermentation processes and in current commercial processes they are the production limiting factors. All these negative aspects can be overcome by the *Chrysosporium* host according to the invention which exhibits much better characteristics than *Trichoderma reesei, Aspergillus niger* and *Aspergillus oryzae* that are commercially used in this respect i.e. exhibits better protein production levels and viscosity properties

and biomass figures.

A *Chrysosporium* strain selected from C1, UV13-6, NG7C-19 and UV18-25 illustrates various aspects of the invention exceedingly well. The invention however also covers recombinant strains or otherwise engineered strains of *Chrysosporium* derived from the four deposited strains that also exhibit any of the novel and inventive characteristics as such or in combination. The deposit data for these strains have been presented elsewhere in the description. The invention also covers recombinant strains or otherwise engineered strains of *Chrysosporium* derived from the four deposited strains that also exhibit any of the novel and inventive characteristics as such or in combination. A *Chrysosporium* strain according to the invention also comprises a strain exhibiting under the corresponding culture conditions a biomass at least twice as low as that of *Trichoderma reesei*, suitably even more up to 5 times lower than that of *Trichoderma reesei*, specifically of a *Trichoderma reesei* exhibiting a viscosity of 200-600 cP as disclosed under the conditions of the examples. A *Chrysosporium* strain according to the invention also comprises a strain producing the polypeptide in at least the amount in moles per liter of cellulase by the strain C1, UV13-6, NG7C-19 or UV18-25 under the corresponding or identical conditions.

Chrysosporium strains according to the invention are further preferred if they exhibit optimal growth conditions at neutral to alkaline pH and temperatures of 25-43°C. A preference can exist for neutral and even for alkaline pH. Such production conditions are advantageous to a number of polypeptides and proteins, in particular those susceptible to attack by acidic pH or those that are inactive or unstable at low temperatures. It is however also an embodiment of the invention to include *Chrysosporium* strains that can be cultured at acidic pH as this can be useful for certain proteins and polypeptides. A suitable acidic pH lies from 7.0. An acidic pH lower than 6.5 is envisaged as providing a good embodiment of the invention. A pH around 5,0-7,0 is also a suitable embodiment. A neutral pH can be 7.0 or around 7 e.g. 6.5-7.5. As stated elsewhere the pH of optimal interest depends on a number of factors that will be apparent to the person skilled in the art. A pH higher than 7.5 is alkaline, suitably between 7.5-9.0 can be used.

When comparing data of strains according to the invention with other strains perhaps having other optimal conditions (e.g. *Aspergillus* and *Trichoderma*) for viscosity measurements, biomass determination or protein production comparisons should be made using the relevant optimal conditions for the relevant strain. This will be obvious to the person skilled in the art.

A *Chrysosporium* strain according to any of the above-mentioned embodiments of the invention, said strain further exhibiting production of one or more of the fungal enzymes selected from the carbohydrate-degrading enzymes, proteases, other hydrolases, oxidoreductase, and transferases mentioned above is considered a particularly useful embodiment of the invention. The most interesting products are specifically cellulases, xylanases, pectinases, lipases and proteases. Also useful as embodiment of the invention however is a *Chrysosporium* strain exhibiting production of

one or more fungal enzymes that exhibit neutral or alkaline optimal stability and/or activity, preferably alkaline optimal stability and/or activity, said enzyme being selected from carbohydrate-degrading enzymes, hydrolases and proteases, preferably hydrolases and carbohydrate-degrading enzymes. In the case of non-recombinant *Chrysosporium*, such enzymes are suitably other than cellulase as disclosed in WO 98/15633. Enzymes of particular interest are xylanases, proteases, esterases, alpha galactosidases, beta-galactosidases, beta-glucanases and pectinases. The enzymes are not limited to the aforementioned. The comments vis-à-vis stability and activity elsewhere in the description are valid here also.

The invention also covers a method of producing a polypeptide of interest, said method comprising culturing a *Chrysosporium* strain in any of the embodiments according to the invention under conditions permitting expression and preferably secretion of the polypeptide and recovering the subsequently produced polypeptide of interest.

Where protein or polypeptide is mentioned, variants and mutants e.g. substitution, insertion or deletion mutants of naturally occurring proteins are intended to be included that exhibit the activity of the non-mutant. The same is valid vis-à-vis the corresponding nucleic acid sequences. Processes such as gene shuffling, protein engineering and directed evolution site directed mutagenesis and random mutagenesis are processes through which such polypeptides, variants or mutants can be obtained. US 5,223,409, US 5,780,279 and US 5,770,356 provide teaching of directed evolution. Using this process a library of randomly mutated gene sequences created for example by gene shuffling via error prone PCR occurs in any cell type. Each gene has a secretion region and an immobilising region attached to it such that the resulting protein is secreted and stays fixed to the host surface. Subsequently conditions are created that necessitate the biological activity of the particular polypeptide. This occurs for a number of cycles ultimately leading to a final gene with the desired characteristics. In other words a speeded up directed process of evolution. US 5,763,192 also describes a process for obtaining DNA, RNA, peptides, polypeptides or protein by way of synthetic polynucleotide coupling stochastically generated sequences, introduction thereof into a host followed by selection of the host cell with the corresponding predetermined characteristic.

Standard cloning and protein or polypeptide isolation techniques can be used to arrive at the required sequence information. Parts of known sequences can be used as probes to isolate other homologues in other genera and strains. The nucleic acid sequence encoding a particular enzyme activity can be used to screen a *Chrysosporium* library for example. A person skilled in the art will realise which hybridisation conditions are appropriate. Conventional methods for nucleic acid hybridisation construction of libraries and cloning techniques are described in Sambrook et al (Eeds) (1989) In "Molecular Cloning. A Laboratory Manual" Cold Spring Harbor, Press Plainview, New York, and Ausubel et al (Eds) "Current Protocols in Molecular Biology" (1987) John Wiley and Sons, New York. The relevant information can also be derived from later handbooks and patents, as well as

from various commercially available kits in the field.

In an alternative embodiment, said method comprises culturing a strain according to the invention under conditions permitting expression and preferably secretion of the protein or polypeptide or precursor thereof and recovering the subsequently produced polypeptide and optionally subjecting the precursor to additional isolation and purification steps to obtain the polypeptide of interest. Such a method may suitably comprise a cleavage step of the precursor into the polypeptide or precursor of interest. The cleavage step can be cleavage with a Kex-2 like protease, any basic amino acid paired protease or Kex-2 for example when a protease cleavage site links a well secreted protein carrier and the polypeptide of interest. A person skilled in the art can readily find Kex-2-like protease sequences as consensus sequence details for such are available and a number of alternatives have already been disclosed e.g. furin.

Suitably in a method for production of the polypeptide according to any of the embodiments of the invention the cultivation occurs at pH higher than 5, preferably 5-10, more preferably 6-9. Suitably in such a method the cultivation occurs at a temperature between 25-43 °C, preferably 30-40°C. The *Chrysosporium* strain used in the method according to the invention is quite suitably a recombinant *Chrysosporium* strain according to any of the embodiments disclosed. The method according to the invention in such a case can further be preceded by the step of production of a recombinant *Chrysosporium* strain according to the invention. The selection of the appropriate conditions will depend on the nature of the polypeptide to be expressed and such selection lies well within the realm of normal activity of a person skilled in the art.

The method of production of a recombinant *Chrysosporium* strain according to the invention is also part of the subject invention. The method comprises stably introducing a nucleic acid sequence encoding a heterologous or homologous polypeptide into a *Chrysosporium* strain, said nucleic acid sequence being operably linked to an expression regulating region, said introduction occurring in a manner known per se for transforming filamentous fungi. As stated above numerous references hereof are available and a small selection has been cited. The information provided is sufficient to enable the skilled person to carry out the method without undue burden. The method comprises introduction of a nucleic acid sequence comprising any of the nucleic acid elements described in the various embodiments of the recombinant *Chrysosporium* according to the invention as such or in combination.

By way of example the introduction can occur using the protoplast transformation method. The method is described in the examples. Alternative protoplast or spheroplast transformation methods are known and can be used as have been described in the prior art for other filamentous fungi. Details of such methods can be found in many of the cited references and are thus incorporated by reference. A method according to the invention suitably comprises using a non-recombinant strain of *Chrysosporium* according to the invention as starting material for introduction of the desired

sequence encoding the polypeptide of interest.

The subject invention also covers a method of producing *Chrysosporium* enzyme, said method comprising culturing a *Chrysosporium* strain according to any of the embodiments of the invention as described above in or on a cultivation medium at pH higher than 5, preferably 5-10, more preferably 6-9, suitably 6-7.5, 7.5-9 as examples of neutral and alkaline pH ranges.

The subject invention also covers such a method using a cultivation medium at a temperature between 25-43 °C, preferably 30-40°C. The combination of preferred pH and temperature is an especially preferred embodiment of the method of producing *Chrysosporium* enzyme according to the invention.

More in general the invention further covers a method of producing enzymes exhibiting neutral or alkaline optimal activity and/or stability, preferably alkaline optimal activity and/or stability. The preferred ranges vis-à-vis pH and optimal activity as well as assays with which to determine such have been provided elsewhere in the description. The enzyme should be selected from carbohydrate-degrading enzymes, proteases, other hydrolases, oxidoreductases, and transferases, as described above, said method comprising cultivating a host cell transformed or transfected with the corresponding enzyme-encoding nucleic acid sequence. Suitably such an enzyme will be a *Chrysosporium* enzyme. A suitable method such as this comprises production specifically of cellulase, xylanase, pectinase, lipase and protease, wherein cellulase and xylanase cleave β-1,4-bonds and cellulase comprises endoglucanase, cellobiohydrolase and β-glucosidase. The method according to the invention can comprise cultivating any *Chrysosporium* host according to the invention comprising nucleic acid encoding such aforementioned enzymes. Suitably the production of non-recombinant *Chrysosporium* hosts according to the invention is directed at production of carbohydrate degrading enzymes, hydrolases and proteases. In such a case the enzyme is suitably other than a cellulase. Suitable examples of products to be produced are given in Tables A and B. Methods of isolating are analogous to those described in WO 98/15633 and are incorporated by reference.

The enzymes produced by *Chrysosporium* strains according to the invention are also covered by the invention. Enzymes of *Chrysosporium* origin as can be isolated from non-recombinant *Chrysosporium* strains according to the invention are also covered. They exhibit the aforementioned stability, activity characteristics. Suitably they are stable in the presence of LAS. In particular proteases with pI 4-9.5, proteases with a MW of 25-95 kD, xylanases with pI between 4.0 and 9.5, xylanases with MW between 25 and 65 kD, endoglucanases with a pI between 3.5 and 6.5, endo-glucanases with MW of 25-55 kDa, ß-glucosidases, α,ß-galactosidases with a pI of 4-4.5, ß-glucosidases, α,ß-galactosidases with a MW of 45-50 kDa, cellobiohydrolases of pI 4-5, cellobiohydrolases of MW 45-60 kDa, e.g. a MW of 55 kD and pI 4.4, polygalacturonases, with a pI of 4.0-5.0 polygalacturonase of 60-70 kDa, e.g. 65 kDa, esterases with a pI 4-5, and esterases with a MW of 95-105 kDa with the afore-mentioned stability, activity characteristics are claimed. The

molecular weights (MW) are those determined by SDS-PAGE. The non-recombinant i.e. natively occurring enzyme is other than cellulase as disclosed in WO 98/15633. An enzyme as disclosed in WO 98/15633 is excluded. Enzymes according to the invention are represented by the enzymes of Table B. Enzymes with combinations of the pI values and molecular weights mentioned above are also covered.

The invention is also concerned with the (over)production of non-protein products by the mutant (recombinant) strains of the invention. Such non-protein products include primary metabolites such as organic acids, amino acids, and secondary such as antibiotics, e.g. penicillins and cephalosporins. These products are the result of combinations of biochemical pathways, involving several fungal genes of interest. Fungal primary and secondary metabolites and procedures for producing these metabolites in fungal organisms are well known in the art. Examples of the production of primary metabolites have been described by Mattey M., The Production of Organic Acids, *Current Reviews in Biotechnology*, 12, 87-132 (1992). Examples of the production of secondary metabolites have been described by Penalva et al. The Optimization of Penicillin Biosynthesis in Fungi, *Trends in Biotechnology* 16, 483-489 (1998).

EXAMPLES

EXAMPLES OF BIOMASS AND VISCOSITY DETERMINATIONS

The following operating parameter data ranges have been determined for fungal fermentations using three different fungal organisms. The three fungal organisms compared are: *Trichoderma longibrachiatum* (formerly *T. reesei*), *Aspergillus niger* and *Chrysosporium lucknowense* (UV18-25).

Viscosity:

Viscosity is determined on a Brookfield LVF viscometer using the small sample adapter and spindle number 31.

Turn the water-circulating pump on 5 minutes prior to viscometer use to equilibrate the water jacket. The water bath temperature should be 30°C.

Obtain a fresh sample of fermentation broth and place 10 ml of the broth in the small sample spindle. Select the spindle speed to give a reading in the range 10-80. Wait four (4) minutes and take the reading from the viscometer scale. Multiply the reading by the factor given below to get the viscosity in centipoise (cP).

Spindle Speed	Multiplication Factor
6	50
12	25
30	10
60	5

The following viscosity ranges have been determined for fermentations using the specified fungal

organism using the above procedure:

	Viscosity in cP
T. longibrachiatum	200 - 600
A. niger	1,500 - 2,000
C. lucknowense (UV18-25)	LT 10

Biomass:

Biomass is determined by the following procedure:

Preweigh 5.5 cm filter paper (Whatman 54) in an aluminium weighing dish.

Filter 5.0 ml whole broth through the 5.5 cm paper on a Buchner funnel, wash the filter cake with 10 ml deionised water, place the washed cake and filter in a weighing pan and dry overnight at 60°C. Finish drying at 100°C for 1 hour, then place in desiccator to cool.

Measure the weight of dried material. Total biomass (g/l) is equal to the difference between the initial and finals weights multiplied by 200.

The following biomass ranges have been determined for fermentations using the specified fungal organism using the above procedure:

	Biomass in g/l
T. longibrachiatum	2.5 - 5
A. niger	5 - 10
C. lucknowense (UV18-25)	0.5 - 1

Protein:

Protein levels were determined using the BioRad Assay Procedure from Sigma Company. Protein levels were highest for the *Chrysosporium*.

The data presented above represent values determined 48 hours into the fermentation process until fermentation end; All values of *Aspergilli* and *Trichoderma* are for commercially relevant fungal organisms and reflect actual commercial data.

A fungal strain such as *C. lucknowense* (UV18-25) has the advantage that the low viscosity permits the use of lower power input and/or shear the in the fermentation to meet oxygen demands for those cases where shear stress on the product may be detrimental to productivity due to physical damage of the product molecule. The lower biomass production at high protein production indicates a more efficient organism in the conversion of fermentation media to product. Thus the *Chrysosporium* provides better biomass and viscosity data whilst also delivering at least as much protein, and in fact a lot more protein than the two commercially used systems which obviously are better than for typically deposited *Aspergillus* or *Trichoderma reesei* strains in general public collections.

The high protein production with low biomass concentration produced by *C. lucknowense*

(UV18-25) would allow development of fermentation conditions with higher multiples of increase in biomass, if increasing biomass results in increased productivity, for the desired product before reaching limiting fermentation conditions. The present high levels of biomass and viscosity produced by the *T. longibrachiatum* and *A. niger* organisms restrict the increase of biomass as the present levels of biomass and viscosity are near limiting practical fermentation conditions.

EXAMPLES OF TRANSFORMATION COMPARING CHRYSOSPORIUM, TRICHODERMA AND TOLYPOCLADIUM GEODES

Two untransformed *Chrysosporium* C1 strains and one *Trichoderma reesei* reference strain were tested on two media (Gs pH 6,8 and Pridham agar, PA, pH 6,8). To test the antibiotic resistance level spores were collected from 7 day old PDA plates. Selective plates were incubated at 32°C and scored after 2,4 and 5 days. It followed that the C-1 strains NG7C-19 and UV18-25 clearly have a low basal resistance level both to phleomycin and hygromycin. This level is comparable to that for a reference *T. reesei* commonly used laboratory strain. Thus there is clear indication these two standard fungal selectable markers can be used well in *Chrysosporium* strains. Problems with other standard fungal selectable markers should not be expected.

Selection of Sh-ble (phleomycin-resistance) transformed *Chrysosporium* strains was succesfully carried out at 50 µg/ml. This was also the selection level used for *T. reesei* thus showing that differential selection can be easily achieved in *Chrysosporium*. The same comments are valid for transformed strains with hygromycin resistance at a level of 150 µg/ml.

Table C

	Gs (pH 6.8)			Pridham Agar (PA,pH 6.8)		
	NG7C-19	UV18-25	T.r.11D5	NG7C-19	UV18-25	T.r.11D5
Phleomycin	7.5 µg/ml	10 µg/ml	5-7.5 µg/ml	2.5 µg/ml	10 µg/ml	2.5 µg/ml
Hygromycin	7.5 - 10 µg/ml	10 µg/ml	10 µg/ml	15 µg/ml	25 µg/ml	15 µg/ml

The protoplast transformation technique was used on *Chrysosporium* based on the most generally applied fungal transformation technology. All spores from one 90mm PDA plate were recovered in 8ml IC1 and transferred into a shake flask of 50ml IC1 medium for incubation for 15 hours at 35°C and 200 rpm. After this the culture was centrifuged, the pellet was washed in MnP, brought back into solution in 10ml MnP and 10mg/ml Caylase C_3 and incubated for 30 minutes at 35°C with agitation (150 rpm).

The solution was filtered and the filtrate was subjected to centrifugation for 10 minutes at 3500 rpm. The pellet was washed with 10 ml $MnPCa^{2+}$. This was centrifuged for 10 minutes at 25°C. Then 50 microlitres of cold MPC was added. The mixture was kept on ice for 30 minutes whereupon 2,5 ml PMC was added. After 15 minutes at room temperature 500 microlitres of the treated protoplasts were mixed to 3 ml of MnR Soft and immediately plated out on a MnR plate containing phleomycin or hygromycin as selection agent. After incubation for five days at 30°C transformants were analysed (clones become visible after 48 hours). Transformation efficiency was determined using 10 microgrammes of reference plasmid pAN8-1[19]. The results are presented in the following Table D.

Table D: Transformation efficiency (using 10 µg of reference plasmid pAN8-1)

	T. reesei	**NG7C-19**	**UV18-25**
Viability	10^6/200 µl	5 10^6 /200 µl	5 10^6 / 200 µl
Transformants Per 200 µl	2500	10^4	10^4
Transformants per 10^6 viable cells	2500	2000	2000

The results show that the *Chrysosporium* transformants viability is superior to that of *Trichoderma*. The transformability of the strains is comparable and thus the number of transformants obtained in one experiment lies 4 times higher for *Chrysosporium* than for *T. reesei*. Thus the *Chrysosporium* transformation system not only equals the commonly used *T. reesei* system, but even outperforms it. This improvement can prove especially useful for vectors that are less transformation efficient than pAN8-1. Examples of such less efficient transformation vectors are protein carrier vectors for production of non-fungal proteins which generally yield 10 times fewer transformants.

A number of other transformation and expression vectors were constructed with homologous *Chrysosporium* protein encoding sequences and also with heterologous protein encoding sequences for use in transformation experiments with *Chrysosporium*. The vector maps are provided in the figures 6-11.

The homologous protein to be expressed was selected from the group of cellulases produced by *Chrysosporium* and consisted of endoglucanase 6 which belongs to family 6 (MW 43 kDa) and the heterologous protein was endoglucanase 3 which belongs to family 12 (MW 25 kDa) of *Penicillium*.

pF6g comprises *Chrysosporium* endoglucanase 6 promoter fragment linked to endoglucanase 6 signal sequence in frame with the endoglucanase 6 open reading frame followed by the endoglucanase 6 terminator sequence. Transformant selection is carried out by using cotransformation

with a selectable vector.

pUT1150 comprises *Trichoderma reesei* cellobiohydrolase promoter linked to endoglucanase 6 signal sequence in frame with the endoglucanase 6 open reading frame followed by the *T. reesei* cellobiohydrolase terminator sequence. In addition this vector carries a second expression cassette with a selection marker i.e. the phleomycin resistance gene (Sh-ble gene).

pUT1152 comprises *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase A promoter linked to endoglucanase.6 signal sequence in frame with the endoglucanase 6 open reading frame followed by the *A. nidulans* anthranilate synthase (trpC) terminator sequence. In addition this vector carries a second expression cassette with a selection marker i.e. the phleomycin resistance gene (Sh-ble gene).

pUT1155 comprises *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase A promoter linked to *Trichoderma reesei* cellobiohydrolase signal sequence in frame with the carrier protein Sh-ble which in turn is linked in frame to the endoglucanase 6 open reading frame followed by the *A. nidulans* trpC terminator sequence. This vector uses the technology of the carrier protein fused to the protein of interest which is known to very much improve the secretion of the protein of interest.

pUT1160 comprises *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase A promoter linked to *Trichoderma reesei* cellobiohydrolase signal sequence in frame with the carrier protein Sh-ble which in turn is linked in frame to the endoglucanase 3 open reading frame of *Penicillium* followed by the *A. nidulans* trpC terminator sequence.

pUT1162 comprises *Trichoderma reesei* cellobiohydrolase promoter linked to endoglucanase 3 signal sequence in frame with the endoglucanase 3 open reading frame of *Penicillium* followed by the *T. reesei* cellobiohydrolase terminator sequence. In addition this vector carries a second expression cassette with a selection marker i.e. the phleomycin resistance gene (Sh-ble gene).

Table E: Comparative transformations

Vector	Strain	Transformation	No of transf.	Tested in liquid culture
PUT1150	UV18-25	selection phleo	285	5
	T. geodes	selection phleo	144	5
PUT1152	UV18-25	cotransformation pAN8.1	398	5
	T. geodes	cotransformation pAN8.1	45	4
PF6g	UV18-25	cotransformation pAN8.1	252	6
	T. geodes	cotransformation pAN8.1	127	5
PUT1162	UV18-25	selection phleo	>400	
	T. geodes	Not done yet		

Table E shows the results of transformation of both *Chrysosporium* UV18-25 and *Tolypocladium geodes*. The transformation protocol used is described in the section for heterologous transformation.

EXAMPLES OF HETEROLOGOUS AND HOMOLOGOUS EXPRESSION OF CHRYSOSPORIUM TRANSFORMANTS

C1 strains (NG7C-19 and/or UV18-25) have been tested for their ability to secrete various heterologous proteins: a bacterial protein (*Streptoalloteichus hindustanus* phleomycin-resistance protein, Sh ble), a fungal protein (*Trichoderma reesei* xylanase II, XYN2) and a human protein (the human lysozyme, HLZ).

The details of the process are as follows:

[1] C1 secretion of Streptoalloteichus hindustanus phleomycin-resistance protein (Sh ble).

C1 strains NG7C-19 and UV18-25 have been transformed by the plasmid pUT720 [1]. This vector presents the following fungal expression cassette:

- *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (gpdA) promoter [2]
- A synthetic *Trichoderma reesei* cellobiohydrolase I (cbh1) signal sequence [1,3]
- *Streptoalloteichus hindustanus* phleomycin-resistance gene Sh ble [4]
- *Aspergillus nidulans* tryptophan-synthase (*trpC*) terminator [5]

The vector also carries the beta-lactamase gene (*bla*) and *E. coli* replication origin from plasmid pUC18 [6]. The detailed plasmid map is provided in figure 2.

C1 protoplasts were transformed according to Durand et al. [7] adapted to C1 (media & solutions composition is given elsewhere): All spores from one 90mm PDA plate of untransformed C1 strain were recovered in 8ml IC1 and transferred into a shake flask with 50ml IC1 medium for incubation 15 hours at 35°C and 150 rpm. Thereupon, the culture was spun down, the pellet washed in MnP, resolved in 10ml MnP + 10mg/ml Caylase C_3, and incubated 30 min at 35°C with agitation (150 rpm). The solution was filtrated and the filtrate was centrifuged 10 min at 3500 rpm. The pellet was washed with 10ml $MnPCa^{2+}$. This was spun down 10min at 3500 rpm and the pellet was taken up into 1ml $MnPCa^{2+}$. 10µg of pUT720 DNA were added to 200µl of protoplast solution and incubated 10min at room temperature (~20°C). Then, 50µl of cold MPC was added. The mixture was kept on ice for 30min whereupon 2.5ml PMC was added. After 15min at room temperature 500µl of the treated protoplasts were mixed to 3ml of MnR Soft and immediately plated out on a MnR plate containing phleomycin (50µg/ml at pH6.5) as selection agent. After 5 days incubation at 30°C, transformants were analysed (clones start to be visible after 48 hours).

The Sh ble production of C1 transformants (phleomycin-resistant clones) was analysed as

follows: Primary transformants were toothpicked to GS+phleomycin (5µg/ml) plates and grown for 5 days at 32°C for resistance verification. Each validated resistant clone was subcloned onto GS plates. Two subclones per transformant were used to inoculate PDA plates in order to get spores for liquid culture initiation. The liquid cultures in IC1 were grown 5 days at 27°C (shaking 200 rpm). Then, the cultures were centrifuged (5000g, 10min.) and 500µl of supernatant were collected. From these samples, the proteins were precipitated with TCA and resuspended in Western Sample Buffer to 4 mg/ml of total proteins (Lowry Method [8]). 10µl (about 40µg of total proteins) were loaded on a 12% acrylamide/SDS gel and run (BioRad Mini Trans-Blot system). Western blotting was conducted according to BioRad instructions (Schleicher & Schull 0.2µm membrane) using rabbit anti-Sh ble antiserum (Cayla Cat. Ref. #ANTI-0010) as primary antibody.

The results are shown in Figure 1 and Table F:

Table F: Sh ble estimated production levels in C1

	Estimated Sh ble quantity on the Western blot	Estimated Sh ble concentration in the production media
Untransformed NG7C-19	Not detectable	
NG7C-19::720 clone 4-1	25 ng	0.25 mg/l
NG7C-19::720 clone 5-1	25 ng	0.25 mg/l
NG7C-19::720 clone 2-2	250 ng	2.5 mg/l
Untransformed UV18-25	Not detectable	
UV18-25::720 clone 1-2	500 ng	5 mg/l
UV18-25::720 clone 3-1	250 ng	2.5 mg/l

These data show that:

1) The heterologous transcription/translation signals from pUT720 are functional in *Chrysosporium*.

2) The heterologous signal sequence of pUT720 is functional in *Chrysosporium*.

3) *Chrysosporium* can be used a host for the secretion of an heterologous bacterial protein.

[2] C1 secretion of the human lysozyme (HLZ).

C1 strains NG7C-19 and UV18-25 have been transformed by the plasmid pUT970G [9]. This vector presents the following fungal expression cassette:

- *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter [2]
- A synthetic *Trichoderma reesei* cellobiohydrolase I (*cbh1*) signal sequence [1,3]
- *Streptoalloteichus hindustanus* phleomycin-resistance gene Sh ble [4] used as carrier-protein

- *Aspergillus niger* glucoamylase *(glaA2)* hinge domain cloned from plasmid pAN56-2 [11, 12]
- A linker peptide (LGERK) featuring a KEX2-like protease cleavage site [1]
- A synthetic human lysozyme gene (*hlz*) [10]
- *Aspergillus nidulans* tryptophan-synthase (*trpC*) terminator [5]

The vector also carries the beta-lactamase gene (*bla*) and *E. coli* replication origin from plasmid pUC18 [6]. The detailed plasmid map is provided in figure 3.

C1 protoplasts were transformed with plasmid pUT970G following the same procedure already described in example 1. The fusion protein (Sh ble :: GAM hinge :: HLZ) is functional with respect to the phleomycin-resistance thus allowing easy selection of the C1 transformants. Moreover, the level of phleomycin resistance correlates roughly with the level of *hlz* expression.

The HLZ production of C1 transformants (phleomycin-resistant clones) was analysed by lysozyme-activity assay as follow: Primary transformants were toothpicked to GS+phleomycin (5µg/ml) plates (resistance verification) and also on LYSO plates (HLZ activity detection by clearing zone visualisation [1, 10]). Plates were grown for 5 days at 32°C. Each validated clone was subcloned onto LYSO plates. Two subclones per transformant were used to inoculate PDA plates in order to get spores for liquid culture initiation. The liquid cultures in IC1 were grown 5 days at 27°C (shaking 180 rpm). Then, the cultures were centrifuged (5000g, 10min.). From these samples, lysozyme activity was measured according to Mörsky et al. [13].

Table G: Active HLZ production levels in C1

	Active HLZ concentration in culture media
Untransformed NG7C-19	0 mg/l
NG7C-19::970G clone 4	4 mg/l
NG7C-19::970G clone 5	11 mg/l
Untransformed UV18-25	0 mg/l
UV18-25::970G clone 1	8 mg/l
UV18-25::970G clone 2	4 mg/l
UV18-25::970G clone 3	2 mg/l
UV18-25::970G clone 2	2.5 mg/l

These data show that:

1) Points 1 & 2 from example 1 are confirmed.
2) Sh ble is functional in *Chrysosporium* as resistance-marker.

3) Sh ble is functional in *Chrysosporium* as carrier-protein.

4) The KEX2-like protease cleavage site is functional in *Chrysosporium* (otherwise HLZ wouldn't be active).

5) *Chrysosporium* can be used as host for the secretion of a heterologous mammalian protein.

[3] C1 secretion of Trichoderma reesei xylanase II (XYN2).

C1 strain UV18-25 has been transformed by the plasmids pUT1064 and pUT1065.

pUT1064 presents the two following fungal expression cassettes:

The first cassette allows the selection of phleomycin-resistant transformants:

- *Neurospora crassa* cross-pathway control gene 1 (*cpc-1*) promoter [14]
- *Streptoalloteichus hindustanus* phleomycin-resistance gene *Sh ble* [4]
- *Aspergillus nidulans* tryptophan-synthase (*trpC*) terminator [5]

The second cassette is the xylanase production cassette:

-*T. reesei* strain TR2 *cbh1* promoter [15]

-*T. reesei* strain TR2 *xyn2* gene (including its signal sequence) [16]

-*T. reesei* strain TR2 *cbh1* terminator [15]

The vector also carries an *E. coli* replication origin from plasmid pUC19 [6]. The plasmid detailed map is provided in figure 4.

pUT1065 presents the following fungal expression cassette:

- *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter [2]
- A synthetic *T. reesei* cellobiohydrolase I (*cbh1*) signal sequence [1,3]
- *S. hindustanus* phleomycin-resistance gene *Sh ble* [4] used as carrier-protein [10]
- A linker peptide (SGERK) featuring a KEX2-like protease cleavage site [1]
- *T. reesei* strain TR2*xyn2* gene (without signal sequence) [16]
- *A. nidulans* tryptophan-synthase (*trpC*) terminator [5]

The vector also carries the beta-lactamase gene (*bla*) and an *E. coli* replication origin from plasmid pUC18 [6]. The plasmid detailed map is provided in figure 5.

C1 protoplasts were transformed with plasmid pUT1064 or pUT1065 following the same procedure already described in example 1. The fusion protein in plasmid pUT1065 (Sh ble :: XYN2) is functional with respect to the phleomycin-resistance thus allowing easy selection of the C1 transformants. Moreover, the level of phleomycin resistance correlates roughly with the level of *xyn2* expression. In pUT1064, *xyn2* was cloned with its own signal sequence.

The xylanase production of C1 transformants (phleomycin-resistant clones) was analysed by xylanase-activity assay as follow: Primary transformants were toothpicked to GS+phleomycin (5µg/ml) plates (resistance verification) and also on XYLAN plates (xylanase activity detection by

clearing zone visualisation [17]). Plates were grown for 5 days at 32°C. Each validated clone was subcloned onto XYLAN plates. Two subclones per transformant were used to inoculate PDA plates in order to get spores for liquid culture initiation. The liquid cultures in IC1+ 5g/l KPhtalate were grown 5 days at 27°C (shaking 180 rpm). Then, the cultures were centrifuged (5000g, 10min.). From these samples, xylanase activity was measured by DNS Technique according to Miller et al. [18]

Table H: Active XYN2 production levels in C1 (best producers)

	Active xylanase II concentration in culture media	Xylanase II specific activity in culture media
Untransformed UV18-25	3.9 U./ml	3.8 U./mg total prot.
UV18-25::1064 clone 7-1	4.7 U./ml	4.7 U./mg total prot.
UV18-25::1064 clone 7-2	4.4 U./ml	4.3 U./mg total prot.
UV18-25::1065 clone 1-1	29.7 U./ml	25.6 U./mg total prot.
UV18-25::1065 clone 1-2	30.8 U./ml	39.4U./mg total prot.

These data show that:

1) Points 1 to 4 from example 2 are confirmed.

2) C1 can be used as host for the secretion of a heterologous fungal protein.

[4] We also illustrate data from expression of transformed UV18-25 wherin the table I shows the results for the plasmids with which transformation was carried out. The Table shows good expression levels for endoglucanase and cellobiohydrolase using heterologous expression regulating sequences and signal sequences but also with homologous expression regulating sequences and signal sequences. The details of the various plasmids can be derived elsewhere in the description and from the figures. The production occurs at alkaline pH at a temperature of 35°C.

Table I: Expression data of transformed UV18-25 strain

Culture	Total proteins	CMCase		ß-glucanase		pH value
	mg/ml	u/ml	u/mg	u/ml	u/mg	
*UV 18-25	100%	100%	100%	100%	100%	7.90
1150-23	94%	105%	111%	140%	149%	7.90
-30	96%	105%	110%	145%	151%	8.10
1152-3	94%	112%	120%	147%	156%	7.85
-4	100%	105%	105%	132%	132%	7.90
1160-2	69%	81%	118%	90%	131%	7.90
-4	73%	72%	98%	83%	114%	8.35
-1	92%	95%	103%	120%	130%	8.45
1162-1	102%	105%	103%	145%	142%	8.20
-11	112%	109%	98%	115%	103%	8.20
F6g-20	104%	102%	98%	130%	125%	7.90
-25	-	-	-	-	-	-

Culture conditions (shake flask): 88h, 35°C, 230 rpm

* all above figures are in relative % to parent UV18-25 strain

Appendix to the Examples: Media

Transformation media:

Mandels Base:	**MnP Medium :**	
KH_2PO_4	2.0 g/l	Mandels Base with
$(NH_4)_2SO_4$	1.4 g/l	Peptone 1 g/l
$MgSO_4.7H_2O$	0.3 g/l	MES 2 g/l
$CaCl_2$	0.3 g/l	Sucrose 100 g/l
Oligoelements	1.0 ml/l	Adjust pH to 5

MnR	**MnP CA^{2+} :**		
MnP+sucrose	130 g/l	MnP Medium +	
Yeast extract	2.5 g/l	$CaCl_2\ 2H_2O$	50 mM
Glucose	2.5 g/l	Adjust pH to 6.5	
Agar	15 g/l		

MnR Soft : MnR with only 7.5 g/l of agar.

MPC :		
$CaCl_2$	50 mM	pH 5.8
MOPS	10 mM	
PEG	40%	

For selection and culture

GS :		
Glucose	10 g/l	
Biosoyase	5 g/l	[Merieux]
Agar	15 g/l	pH should be 6.8
PDA :		
Potato Dextrose Agar	39 g/l	[Difco]
		pH should be 5.5
MPG :		
Mandels Base with		
K.Phtalate	5 g/l	
Glucose	30 g/l	
Yeast extract	5 g/l	

The regeneration media (MnR) supplemented with 50 µg/ml phleomycin or 100-150 µg/ml hygromycin is used to select transformants. GS medium, supplemented with 5 µg/ml phleomycin is used to confirm antibiotic resistance.

PDA is a complete medium for fast growth and good sporulation. Liquid media are inoculated with 1/20th of spore suspension (all spores from one 90mm PDA plate in 5ml 0.1% Tween). Such cultures are grown at 27°C in shake flasks (200 rpm).

ISOLATION AND CHARACTERISATION OF CI PROTEINS

The process for obtaining various proteins is described as are a number of characteristics of the proteins. The tables A, B and J provide details of purification scheme and activities. Isolation occurs from the *Chrysosporium* culture filtrate using DEAE-Toyopearl ion exchange chromatography analogously to the method described in WO 98/15633, which is incorporated herein by reference. The non-bound fraction (F 60-31 CF) obtained from this chromatography was purified using Macro Prep Q ion exchange chromatography after equilibration to pH 7,6. The non-bound fraction (NBNB) was pooled and bound proteins were eluted in 0-1 M NaCl gradient. The NBNB fraction provided major protein bands of 19, 30, 35 and 46 kD and a minor one of 51 kD. In 0-1 M NaCl gradient protein peaks were eluted from various fractions. 39-41 included 28, 36 and 60 kD proteins, 44-48 included

28, 45 and 66 kD as major protein bands with 33, 36, 55, 60 and 67 kD proteins, the 49-51 fraction gave 30, 36, 56 and 68 kD proteins and the 52-59 fraction included major 33 and 55 kD proteins and minor 28 and 36 kD proteins. The pooled NBNB fraction was further purified by hydrophobic chromatography on Phenyl Superose. The NBNB fraction was equilibrated with 0,03M Na-phosphate buffer pH 7,0 containing 1,2 M $(NH_4)_2SO_4$ and applied to a column. Adsorbed proteins were eluted in 1,2-0,6 M $(NH_4)_2SO_4$ gradient. Thus homogeneous xylanase with MW 30 and 51 kD and pI 9.1 and 8.7 respectively were obtained as was a 30 kD protease with pI 8,9.

The xylanases did not possess MUF cellobiase activity and are thus true xylanases. The alkaline 30 kD xylanase (pI 9.1) possessed high activity within a very broad pH range from 5-8 maintaining 65% of maximum activity at pH 9-10; it is a member of the xylanase F family; its partial nucleotide and amino acid sequences are depicted in SEQ ID No. 5. The partial amino acid sequence depicted corresponds to about amino acids 50-170 from the N terminus of the mature protein. Xylanases according to invention have at least 60%, preferably at least 70%, most preferably at least 80% sequence identity of the partial amino acid sequence of SEQ ID No. 5. The corresponding xylanase promoter, which is a preferred embodiment of the invention, can be identified using the partial nucleotide sequence of SEQ ID No. 5. The 51 kD xylanase (pI 8,7) possessed maximum activity at pH 6 and retained at least 70% of its activity at pH 7,5 and it retained at least 50% of its activity at pH 8,0. It was not very stable with only 15% activity at pH 5,5 and 4% at pH 7,5. The Michaelis constant toward birch xylan was 4,2 g/l for 30kD xylanase and 3,4 g/l for 51 kD xylanase. Temperature optimum was high and equal to 70°C for both xylanases.

The 30 kD protease activity measured towards proteins of the NBNB fraction appeared to be equal to 0,4 x 10_{-3} units/ml at 50°C and pH 7,90 kD. The fraction exhibited activity toward dyed casein of 0,4 arbitrary units/mg (pH 7). Addition of urea as chaotropic agent resulted in 2-3 times increase of protease activity. The effect of the protease on xylanase activity was significant. Only 30% xylanase activity remained at pH 10,3 and 50°C after 30 minutes of incubation. At pH 8 95% of the xylanase activity remained. LAS addition resulted in a dramatic decrease of xylanase activity at pH 8 and 10,3 with only 50% xylanase activity after 10 minutes of incubation with or without protease inhibitor PMSF. The 30 kD protease was alkaline with pH optimum at pH 10-11. The activity is inhibited by phenylmethylsulfonyl fluoride (PMSF) and not by iodoacetic acid, pepstatin A and EDTA which characterises it as a serine type protease. The protease is not active towards C1 proteins at neutral pH and 50°C without chaotropic agents. Increase of pH and the addition of chaotropic agents such as LAS, SDS and urea significantly increase proteolysis.

The 39-41 fraction was purified by hydrophobic chromatography on plenol superose. Fractions were equilibrated with 0,03M Na phosphate buffer pH 7,2 containing 1,5 M $(NH_4)_2SO_4$ and applied to a column. Adsorbed proteins were eluted in 1,5-0 M $(NH_4)_2SO_4$ gradient. Thus homogenous xylanase with MW 60 kD and pI 4,7 was obtained. This xylanase possessed activities

towards xylan, MUF-cellobioside, MUF-xyloside and MUF-lactoside. This xylanase probably belongs to family 10 (family F). This xylanase was stable at pH from 5 to 8 during 24 hours and retained more than 80% activity at 50°C. It retained 70% activity at pH 5-7 at 60°C. It kept 80% activity during 5 hours and 35% during 24 hours at 50°C and pH 9. At pH 10 60% activity was retained at 50°C and 0,5 hours of incubation. After 5 hours of incubation at pH 8 and 60°C 45% activity was found decreasing to 0 after 24 hours. It had a pH optimum within the pH range of 6-7 and kept 70% activity at pH 9 and 50% of its activity at pH 9,5. The Michaelis constant toward birch xylan was 0,5 g/l. Temperature optimum was high and equal to 80°C.

Fraction 44-48 was then purified by chromatofocusing on Mono P. A pH gradient from 7,63-5,96 was used for the elution of the proteins. As a result 45 kD endoglucanase was isolated with a pI of 6. The 45 kD endo had maximum activity at pH 5 toward CMC and at pH 5-7 toward RBB-CMC. The 45 kD endo retained 70% of its maximal activity toward CMC at pH 6,5 and 70% of its maximal activity toward RBB-CMC was retained at pH 7,0; 50% of its maximal activity toward CMC was retained at pH 7 and 50% of its maximal activity toward RBB-CMC was retained at pH 8. The Michaelis constant toward CMC was 4,8 g/l. Temperature optimum was high and equal to 80°C. Other proteins 28, 33, 36, 55, 60 and 66 kD were eluted mixed together.

Fraction 52-58 was purified by chromatofocusing on Mono P too with a pH gradient 7,6-4,5. Individual 55 kD endoglucanase with pI 4,9 was obtained. The 55 kD endo was neutral. It has a broad pH optimum from 4,5-6 and 70% activity was retained at pH 7,0 both for CMC and RBB-CMC and 50% activity was retained at pH 8 for both CMC and RBB-CMC. The Michaelis constant toward CMC was 1 g/l. Temperature optimum was high and equal to 80°C. A number of fractions also held proteins with MW of 28, 33 and 36 kD.

45, 48 and 100 kD proteins were isolated from bound DEAE Toyopearl fraction of F 60-8 UF conc of *Chrysosporium* culture from fractions 50-53 using Macro Prep Q chromatography.

Fraction 50-53 was equilibrated with 0.03 M imidazole HCL buffer, pH 5.75 and was applied to a column and the adsorbed proteins were eluted in 0,1-0,25 M NaCl gradient for 4 h. As a result 45 kD (pI 4.2), 48 kD (pI 4.4) and 100 kD (pI 4.5) proteins were isolated in homogenous states.

The 45 kD is supposedly a alpha beta-galactosidase by virtue of its activity toward p-nitrophenyl alpha-galactoside and p-nitrophenyl beta-galactoside. The pH optimum was 4,5 70% activity was maintained at pH 5,7 and 50% of its activity was retained at pH 6,8. The temperature optimum was 60°C.

The 48 kD protein was a cellobiohydrolase having high activity toward p-nitrophenyl beta-glucoside and also activities toward MUF cellobioside, MUF lactoside and p-nitrophenyl butyrate. The 48 kD protein had a pH optimum of 5 toward CMC and 5-6 toward RBB-CMC.

The 100 kD protein with pI 4,5 possessed activity only toward p-nitrophenyl butyrate. It is probably an esterase but is not a feruloyl esterase as it had no activity against methyl ester of ferulic

acid. It had neutral/alkaline pH optimum (pH 8-9) and optimal temperature of 55-60°C.

The 90 kD protease with pI 4,2 was isolated from the bound fraction and the activity measured towards proteins of the NBNB fraction appeared to be equal to 12 x 10_3 units/ml at 50°C and pH 7,90 kD. The fraction exhibited activity toward dyed casein of 0,01 arbitrary units/mg (pH 7). Addition of urea as chaotropic agent resulted in 2-3 fold increase of protease activity as did addition of LAS at both pH 7 and 9 (50°C). The 90 kD protease was neutral with pH optimum at pH 8. The activity is inhibited by phenylmethylsulfonyl fluoride (PMSF) and not by iodoacetic acid, pepstatin A and EDTA which characterises it as a serine type protease.

Also isolated from the bound fraction were 43 kD endoglucanase with pI 4.2 (fraction 33-37) and 25 kD endoglucanase with pI 4.1 (fraction 39-43), 55 kD cellobiohydrolase with pI 4.9 (fraction 39-43) and 65 kD polygalacturonase with pI 4.4 (fraction 39-43). The endoglucanases did not possess activity towards avicel or MUF cellobioside and possessed high activity toward MC, RBB-CMC, CMC41, beta-glucan and endoglucanase. The 25 kD endo did not produce glucose from CMC and the 43 kD endo did. No glucose was formed from avicel. The pH optimum for the 43 kD protein was 4,5 with 70% maximum activity maintained at pH 7.2 and 50% at pH 8. The 43 kD endo kept 70% activity at pH 5 and 6 during 25 hours of incubation. It kept only 10% at pH 7 during this incubation period. The 25 kD endo had pH optimum of activity at pH 5 toward CMC and broad pH optimum of activity toward RBB-CMC with 70% of the maximum activity being kept at pH 9 and with 50% of the maximum activity being at pH 10. It kept 100% activity at pH 5 and 6 and 80% at pH 7, 8, 8.6 and 9.6 during 120 hours of incubation. The 25 kD endo had a temperature optimum of activity at 70°C. The 43 kD endo had a temperature optimum of activity at 60°C. The Michaelis constants towards CMC were 62 and 12,7 g/l for 25 and 43 kD endo respectively. The poly-galacturonase is a pectinase. The Michaelis constant toward PGA was 3.8 g/l. The pH optimum of PGU activity is within pH range 5-7 and T optimum within 50-65°C.

Genes encoding *C. lucknowense* proteins were obtained using PCR and characterised by sequence analysis. The corresponding full genes were obtained by screening (partial) gene libraries using the isolated PCR fragments. The full gene of the 43 kD endoglucanase (EG6, Family 6) of the C1 strain has been cloned, sequenced and analysed (including 2.5 kb promoter region and 0.5 kb terminator region). Its nucleotide and amino acid sequences are depicted in SEQ ID No. 1. Predicted molecular weight of the mature protein is 39,427 Da and predicted pI is 4.53, which values correspond well with the measured values. Protein alignment analysis with other glycosyl hydrolases of the family 6.2 shows that C1-EG6 does not include a cellulose-binding domain (CBD) Homology analysis using SwissProt SAMBA software (Smith & Waterman algorithm, Gap penalty 12/2, alignment 10, Blosum62 matrix) shows that C1-EG6 has 51.6% identity with *Fusarium oxysporum* EG-B (over 376 amino acids), 51.0% identity with *Agaricus bisporus* CBH3 (over 353 amino acids), and 50.7% identity with *Trichoderma reesei* CBH2 (over 367 amino acids). The putative signal sequence runs

Met 1 to Arg 28. The promoter contains several potential CreA binding sites, so it is very likely that this promoter would be subject to glucose repression in a fungal strain with working CreA regulation.

Similarly, the full gene of the 25 kD endoglucanase (EG5, Family 45) of the C1 strain has been cloned, sequenced and analysed (including 3.3 kb promoter region and 0.7 kb terminator region). The nucleotide and amino acid sequences are depicted in SEQ ID No. 2. Predicted molecular weight of the mature protein is 21,858 Da and predicted pI is 4.66, which values correspond well with the measured values. This is the smallest fungal endoglucanase known to date. Protein alignment analysis with other glycosyl hydrolases of the family 45 shows that C1-EG5 does not include a cellulose-binding domain (CBD), nor a cohesin/dockerin domain. Homology analysis using NCBI-BLASTP2 software (Gap penalty 11/1, alignment 10, Blosum62 matrix) shows that the closest homologous protein to C1-EG5 is *Fusarium oxysporum* EG-K with 63% identity. The putative signal sequence runs Met 1 to Ala 18. The promoter contains many potential CreA binding sites, so it is very likely that this promoter would be subject to glucose repression in a fungal strain with working CreA regulation.

Furthermore, an additional endoglucanase was found by PCR based on family 12 cellulases homology analysis. The partial nucleotide and amino acid sequence of this additional endoglucanase (EG3, Family 12) is given in SEQ ID No. 3.

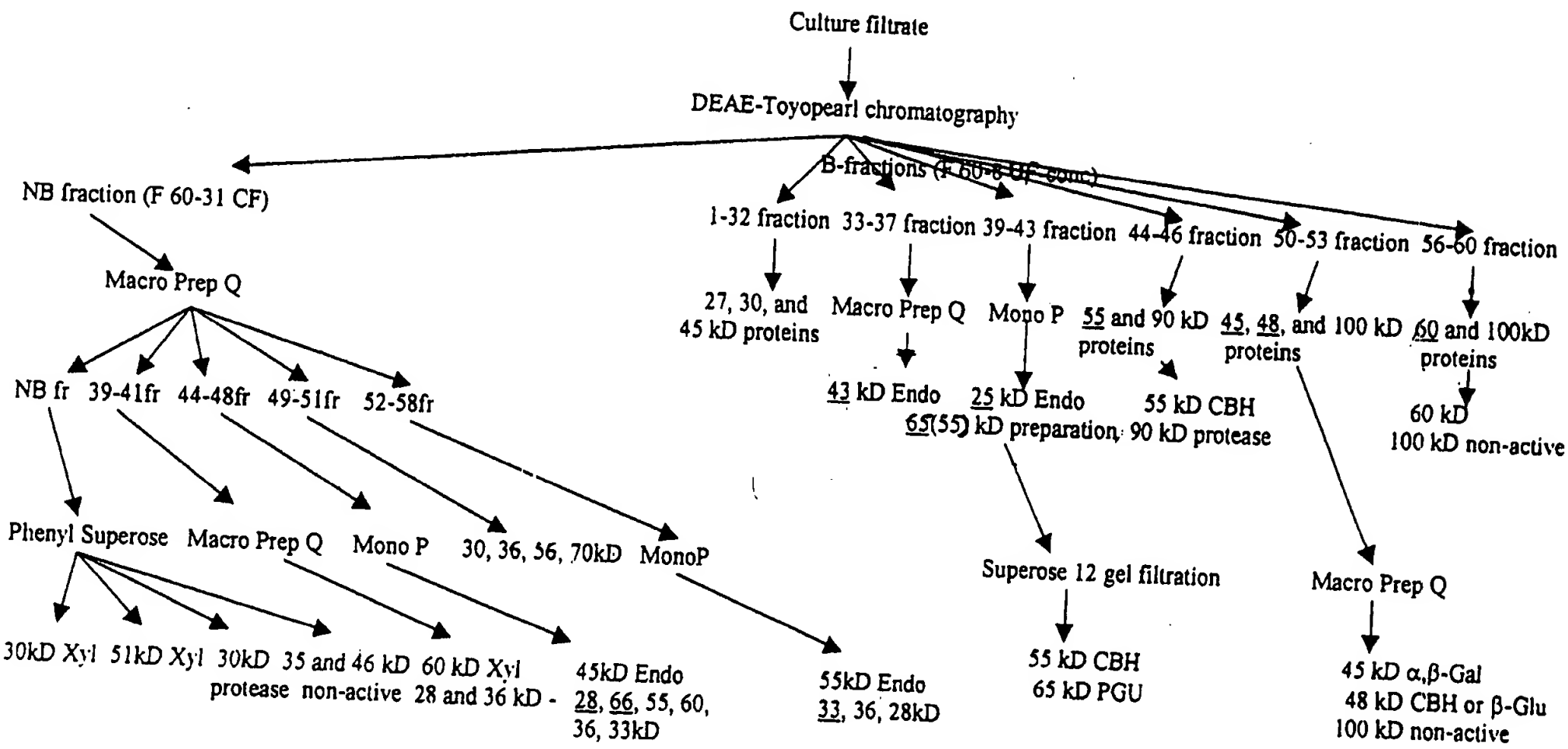
The 55kD protein was a cellobiohydrolase (referred to herein as CBH1) with activity against MUF-cellobioside, MUF lactoside, FP and avicel, also against p-nitrophenyl β-glucoside, cellobiose and p-nitrophenyl lactoside. Its activity toward MUF cellobioside is inhibited by cellobiose. The inhibition constant 0,4 mM was determined. The Michaelis constant toward MUF cellobioside was 0,14 mM, toward MUF lactoside was 4 mM and toward CMC was 3,6 g/l. The pH optimum is rather broad from 4,5 to 7. 50% of maximum activity toward CMC and 80% activity toward RBB-CMC is kept at pH 8. 70-80% activity within pH 5-8 is kept during 25 hours of incubation. The temperature optimum is 60-70°C. CBH1 is probably a member of the cellobiohydrolase family 7; its partial nucleotide and amino acid sequences are depicted in SEQ ID No. 4. The partial amino acid sequence depicted corresponds to about amino acids 300-450 from the N terminus of the mature protein. A cellobiohydrolase according to the invention has at least 60%, preferably at least 70%, most preferably at least 80% sequence identity of the partial amino acid sequence of SEQ ID No. 4. The corresponding CBH promoter, which is a preferred embodiment of the invention, can be identified using the partial nucleotide sequence of SEQ ID No. 4. A synergistic effect was observed between 25 kD endo and 55 kD CBH during avicel hydrolysis. Synergism coefficient was maximal at the ratio of 25 kD endo to 55 kD CBH 80:20. The K_{syn} was 1,3 at its maximum.

Tables A, B and J illustrate the details of the above.

Table J

Purification scheme

Purification of F 60-8 (UF-conc) and F 60-31 CF samples.



The expression level of five main *Chrysosporium* genes was studied by Northern analysis. Various strains of *C. lucknowense* were grown in rich medium containing pharmedia with cellulose and lactose (medium 1) or rich medium containing pharmedia and glucose (medium 2) at 33°C. After 48 h, mycelium was harvested and RNA was isolated. The RNA was hybridised with 5 different probes: EG5, EG6, EG3, XylF and CBH. After exposure, the Northern blots were stripped and hybridised again with a probe for ribosomal L3 as a control for the amount of mRNA on the blot. Most strains showed very high response for CBH and high response for XylF in medium 1; in medium 2, half of the strain showed high response for all genes, and the other half showed low response. The order of expression strength was deducted from these data as CBH > XylF > EG5 > EG3 > EG6.

Tables A, B and J illustrate the details of the above.

Description of the figures

Figure 1 is a Western blot as described in the Examples

Figure 2 is a pUT720 map

Figure 3 is a pUT970G map

Figure 4 is a pUT1064 map

Figure 5 is a pUT1065 map

Figure 6 is a pF6g map

Figure 7 is a pUT1150 map

Figure 8 is a pUT1152 map

Figure 9 is a pUT1155 map

Figure 10 is a pUT1160 map

Figure 11 is a pUT1162 map

Figure 12: Ion exchange chromatography on Macro Prep Q of NB-fraction after DEAE-Toyopearl of F-60-31 CF sample.

Figure 13: pH courses of activities of 30kD (pI 8.9) and 90 kD (pI 4.2) proteases toward C1 proteins (50°C, 30 min. incubation).

Figure 14: Effect of 30 kD (pI 8.9) "alkaline" protease on xylanase activity of the NBNB-fraction (Macro Prep Q) of F 60-31 CF at 50°.

Figure 15: Effect of 90 kD (pI 4.2)"neutral" protease on CMCase activity of the proteins in the bound fraction #44-45 (DEAE-Toyopearl) of F 60-8 UV-conc sample at 50°C.

Figure 16: Complete hydrolysis of polygalacturonic acid by 65 kD polygalacturonase (pI 4.4): 50°C, pH 4.5; concentration pf PGA= 5 g/l, concentration of protein = 0.1 g/l.

Figure 17: pH- and temperature dependencies of polygalacturonase activity of F-60-43 UF-conc.

Figure 18: Inhibition of activity toward MUF-cellobioside by cellobiose for 55 kD CBH (pI 4.4): pH 4.5, 40°C.

Figure 19: Synergistic effect between 25 kD Endo (pI 4.1) and 55 kD CBH (pI 4.4) toward avicel (40°C, pH 5, 25 min).

Figure 20: Complete hydrolysis of CMC (a) and avicel (b) by the enzymes isolated from bound fractions of F-60-8 UF-conc. sample (50°C, pH 5): concentration of CMC and avicel - 5 g/l, concentration of 25 kD Endo = 0.01 g/l, concentration of 43 kD Endo = 0.02 g/l; 1-25 kD Endo (pI 4.1), 2-43 kD Endo (pI 4.2).

Figure 21: Complete hydrolysis of CMC (1) and avicel (2) by 55 kD CBH (pI 4.4) without (a) and with (b) glucono-δ-lactone (50°C, pH 4.5): concentration of CMC and avicel = 5 g/l, concentration of protein = 0.1 g/l, concentration of glucono-δ-lactone = 5 g/l.

Figure 22: pH-Dependence is of CMCase and RBB-CMCase activities of the enzymes isolated from F-60-8 UF-conc. sample: 1-25 kD Endo (pI 4.1), 2-43 kD Endo (pI 4.2).

Figure 23: pH-Dependencies of CMCase and RBB-CMCase activities of 55 kD CBH (pI 4.4).

Figure 24: Temperature dependencies of CMCase activity (pH 4.5) of the enzymes isolated from bound fractions of F-60-8 UF-conc. sample: 1-55 kD CBH (pI 4.4), 2-25 kD Endo (pI 4.1), 3-43 kD Endo (pI 4.2).

Figure 25: pH-stability (50°C) of the enzymes isolated from bound fractions of F-60-8 UF-conc. sample: 1-55 kD CBH (pI 4.4), 2-25 kD Endo (pI 4.1), 3-43 kD Endo (pI 4.2).

Figure 26: Adsorption of the enzymes isolated from bound fractions of F-60-8 UF-conc. sample.

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CLAIMS

1. A mutant *Chrysosporium* strain comprising a nucleic acid sequence encoding a polypeptide of interest, said nucleic acid sequence being operably linked to an expression-regulating region and optionally a secretion signal sequence, said mutant strain expressing said polypeptide of interest at a higher level than the corresponding non-mutant strain under the same conditions.

2. A mutant *Chrysosporium* strain according to claim 1, said mutant being obtained by recombinant methods comprising stable introduction of at least one heterologous nucleic acid sequence selected from heterologous polypeptide-encoding nucleic acid sequences, heterologous signal sequences and heterologous expression-regulating sequences.

3. A mutant *Chrysosporium* strain according to claim 2, wherein said polypeptide of interest is a heterologous polypeptide of plant, animal (including human), algal, bacterial, archaebacterial or fungal origin.

4. A mutant *Chrysosporium* strain according to claim 1 or 2, wherein said polypeptide of interest is a homologous polypeptide which is expressed at a higher level than in the corresponding non-mutant strain under the same conditions.

5. A mutant *Chrysosporium* strain according to any one of claims 1-4, wherein said polypeptide of interest is selected from carbohydrate-degrading enzymes, proteases, lipases, esterases, other hydrolases, oxidoreductases and transferases.

6. A mutant *Chrysosporium* strain according to any one of claims 1-4, wherein said polypeptide of interest is selected from fungal enzymes allowing (over)production of primary metabolites, including organic acids, and secondary metabolites, including antibiotics.

7. A mutant *Chrysosporium* strain according to any one of claims 1-6, wherein said polypeptide of interest is inactivated at a pH below 5, especially at a pH below 6.

8. A mutant *Chrysosporium* strain according to any one of claims 1-7, wherein said polypeptide of interest exhibits optimal activity and/or stability at a pH above 6, and/or has more than 70% of its activity and/or stability at a pH above 6.

9. A mutant *Chrysosporium* strain according to any one of claims 1-8, comprising a heterologous signal sequence, preferably a fungal, e.g. ascomycete, signal sequence.

10. A mutant *Chrysosporium* strain according to claim 9, wherein the fungal signal sequence is a signal sequences of a cellulase, β-galactosidase, xylanase, pectinase, esterase, protease, amylase, polygalacturonase or hydrophobin.

11. A mutant *Chrysosporium* strain according to any one of the preceding claims, further comprising a selectable marker, such as a marker conferring resistance to a drug or relieving a nutritional defect.

12. A mutant *Chrysosporium* strain according to any one of the preceding claims, comprising a heterologous expression-regulating region, preferably a fungal expression-regulating sequence.

13. A mutant *Chrysosporium* strain according to claim 12, wherein the expression-regulating region comprises is an inducible promoter.

14. A mutant *Chrysosporium* strain according to claim 12 or 13, wherein the expression-regulating region comprises a high expression promoter.

15. A mutant *Chrysosporium* strain according to claim 1, said mutant being obtained by mutagenesis steps including at least one of UV irradiation and chemical mutagenesis, preferably comprising a first UV irradiation step, a N-methyl-N'-nitro-N-nitrosoguanidine treatment step and a second UV irradiation step.

16. A mutant *Chrysosporium* strain according to any one of the preceding claims, said mutant being derived from *Chrysosporium lucknowense*, especially from *C. lucknowense* strain C1 (VKM F-3500 D).

17. A mutant *Chrysosporium* strain according to claim 16, said mutant corresponding to or being derived from one of *Chrysosporium lucknowense* mutant strains UV13-6 (VKM F-3632 D), NG7C-19 (VKM F-3633 D), and UV18-25 (VKM F-3631 D).

18. A mutant *Chrysosporium* strain according to any one of the preceding claims, said strain exhibiting a biomass of less than half that of *Trichoderma reesei*, with said *Trichoderma* in culture exhibiting a viscosity of 200-600 cP when cultured under equivalent optimal conditions.

19. A mutant *Chrysosporium* strain according to any one of the preceding claims, said strain producing at least the amount of cellulase in moles per liter as produced by any of the *Chrysosporium lucknowense* mutant strains C1, (VKM F-3500 D), UV13-6 (VKM F-3632 D), NG7C-19 (VKM F-3633 D), and UV18-25 (VKM F-3631 D).

20. A mutant *Chrysosporium* strain according to any one of the preceding claims, said strain producing less protease than produced by the *Chrysosporium lucknowense* strains C1 (VKM F-3500 D), preferably less than half the amount produced by said C1 strain.

21. A nucleic acid construct comprising a nucleic acid expression-regulatory region derived from *Chrysosporium*, preferably from *Chrysosporium lucknowense*, more preferably from *C. lucknowense* C1 (VKM F-3500 D) or UV18-25 (VKM F-3631 D), operably linked to a poly-peptide-encoding nucleic acid sequence.

22. A nucleic acid construct according to claim 21, said expression-regulatory region comprising a promoter sequence associated with cellulase expression or xylanase expression, preferably a cellobiohydrolase (CBH1) promoter sequence.

23. A recombinant microbial strain, preferably a fungal strain, containing a nucleic acid construct according to claim 21 or 22, and capable of expressing the polypeptide encoded by the coding nucleic acid sequence.

24. A method of producing a polypeptide of interest, said method comprising culturing a strain according to any one of claims 1-20 and 23-24 under conditions permitting expression and preferably secretion of the protein or polypeptide and recovering the subsequently produced polypeptide of interest.

25. A method according to claim 24, further comprising a cleavage step of a precursor of said polypeptide into the polypeptide or precursor of interest, preferably a cleavage with a Kex-2 like protease, any basic amino acid paired protease or Kex-2.

26. A method according to claim 24 or 25, wherein the cultivation occurs at pH in the range 6-9, and/or at a temperature between 25 and 43 °C.

27. A method for producing a mutant *Chrysosporium* strain according to any of claims 1-20 comprising stably introducing a nucleic acid sequence encoding a heterologous or homologous polypeptide into a *Chrysosporium* strain, said nucleic acid sequence being operably linked to an expression regulating region, said introduction occurring in a manner known per se for transforming filamentous fungi.

28. A method according to claim 27, wherein the transformation method is the protoplast transformation method.

29. A *Chrysosporium* xylanase of the xylanase F family, having a pI of 9,1, an MW of about 30 kD by SDS PAGE, and having at least 75% amino acid identity over a stretch of 120 amino acids with the amino acid sequence depicted in SEQ ID No. 5.